Function and compartmentalization of circulating versus tissue resident memory T cells

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Abstract

Intensified efforts to promote protective T cell-based immunity in vaccines and immunotherapies have created a compelling need to expand our understanding of human T cell function and maintenance beyond its characterization in peripheral blood. The paradigm that memory T lymphocytes are continuously circulating through the body in search of their cognate antigen has been recently challenged by the discovery of memory T cells residing in a variety of tissues, including the bone marrow (BM). However, the division of labor and lifestyle of circulating versus tissue resident memory T cells remains poorly understood.

Previous work of our group has shown that the human BM is home to a great number of memory T cells. Despite of CD69 expression, BM memory T cells are resting in terms of transcription profile, proliferation and migration. Moreover, BM memory $CD4^+$ T cells contain a wide array of antigen specificities, and show high protective potential, being mostly polyfunctional. Interestingly, memory $CD4^+$ T cells specific for systemic childhood antigens, like measles, mumps or rubella have been found in the BM of elderly humans, even when they were no longer detectable in peripheral blood (PB) circulation. From these results, we hypothesized that BM memory T cells are resident, resting and maintain long-term memory to systemic antigens. The survival mechanisms involved in the maintenance of circulating and BM resident memory T cells; as well as the capacities of tissue resident memory T cells to be reactivated and mobilized into blood circulation after systemic antigen re-challenge to confer us with immune protection remains to be elucidated.

In this doctoral thesis, I have shown that PB and BM memory T cells have different survival capacities, as well as identified the role of soluble factors and direct contact with stromal cells in their maintenance, and the molecular mechanisms underlying. Moreover, using flow cytometric and sequencing analysis of the TCRβ repertoire, I have determined that PB and BM memory T cells are separated cell populations, highly compartmentalized in their respective tissues. Finally, by tracking the dynamics of antigen-specific memory $CD4^+$ T cells after systemic MMR re-vaccination I could show that T_{RM} $CD4^+$ T cells specific for systemic antigens can be rapidly mobilized into blood circulation and contribute to the immune response. Taken together, these studies provide a more comprehensive understanding of the function and maintenance of immunological memory in humans.

Zusammenfassung

Verstärkte Anstrengungen zur Förderung der T-Zell-basierten Immunität in Impfstoffen und Therapien haben eine zwingende Notwendigkeit für unser Verständnis der menschlichen T-Zell-Funktion und -Erhaltung, die über eine Charakterisierung in peripheren Blut hinausgehen, geschaffen. Das Paradigma, dass Gedächtnis-T-Lymphozyten kontinuierlich auf der Suche nach ihrem Antigen durch den Körper zirkulieren wurde vor kurzem durch die Entdeckung der Gedächtnis-T-Zellen, die in einer Vielzahl von Geweben, einschließlich des Knochenmarks (BM) angesiedelt sind, herausgefordert. Allerdings bleibt der Unterschied zwischen Funktionsweise und Lebensstil von zirkulierenden und gewebeansässigen Gedächtnis-T-Zellen nur unzulänglich verstanden.

Vorhergehende Arbeiten unserer Gruppe haben gezeigt, dass das menschliche Knochenmark die Heimat für eine große Anzahl Gedächtnis-T-Zellen ist. Gedächtnis-T-Zellen im Knochenmark sind trotz CD69 Expression hinsichtlich ihres Transkriptionsprofils, der Proliferation und Migration ruhend. Zusätzlich beinhalten $CD4^+$ Gedächtnis-T-Zellen aus dem Knochenmark ein breites Spektrum an Antigenspezifitäten und ein hohes protektives Potential, da sie größtenteils polyfunktional sind. Interessanterweise wurden $CD4^+$ Gedächtnis-T-Zellen spezifisch für systemische Kindheitsantigene wie Masern, Mumps oder Röteln im Knochenmark von älteren Menschen gefunden, auch wenn sie nicht mehr in der peripheren Blutzirkulation nachgewiesen werden konnten. Aus diesen Ergebnissen ziehen wir die Hypothese, dass Gedächtnis-T-Zellen aus dem Knochenmark sesshaft und ruhend sind und das Langzeitgedächtnis gegen systemische Antigene erhalten. Sowohl der Überlebensmechanismus, der den Erhalt von zirkulierenden und knochenmarksansässigen Gedächtnis-T-Zellen ermöglicht, als auch die Kapazität von gewebsansässigen Gedächtnis-T-Zellen nach einer systemischen Herausforderung mit Antigen reaktiviert und in die Blutzirkulation mobilisiert zu werden, um uns Immunschutz zu verleihen, sind bisher nur unzureichend geklärt.

In dieser Dissertation habe ich gezeigt, dass Gedächtnis-T-Zellen aus dem peripheren Blut und Knochenmark unterschiedliche Überlebensfähigkeiten haben. Weiterhin habe ich die Rolle von löslichen Faktoren und dem direkten Kontakt zu Stroma Zellen in ihrer Erhaltung sowie die zugrundeliegenden molekularen Mechanismen identifiziert. Zudem habe ich mittels Durchflusszytometrie und Sequenzanalysen des TCRβ Repertoires bestimmt, dass Gedächtnis-T-Zellen aus dem peripheren Blut und Knochenmark unterschiedliche

Zellpopulationen sind, die stark in ihren jeweiligen Geweben getrennt sind. Schließlich konnte ich durch Verfolgen der Dynamik von antigenspezifischen $CD4^+$ Gedächtnis T-Zellen nach Auffrischen der systemischen MMR Impfung zeigen, dass sesshafte Gedächtnis-T-Zellen, die spezifisch für systemische Antigene sind, schnell in die Blutzirkulation mobilisiert werden und dort zu der Immunreaktion beitragen. Zusammenfassend bieten diese Studien ein umfassenderes Verständnis der Funktion und des Erhalts des immunologischen Gedächtnisses in Menschen.

1 Introduction

1.1 Adaptive T cell immune responses

The immune system is the collection of cells, tissues and molecules that protect the body from numerous pathogens present in our environment. The mammalian immune system can be divided in two main arms: innate and adaptive immunity. The cells and receptors of the innate immune system are critical for the rapid recognition of the infectious agent and initiating a proinflammatory response. The inflammation generated by innate immune cells (neutrophils, macrophages, monocytes, natural killer cells (NKs), dendritic cells (DCs), etc.) is important for the initial containment of the infection, but also for directing the expansion and differentiation of the adaptive immune cells. In response to the inflammatory environment created by the innate immune response, cells from the adaptive immune system (B and T cells) are stimulated to expand in number and to differentiate into lineages of cells with a range of functions needed for the immunological challenge[1].

The activation and programming of T cells from their naïve state to an effector state is critical to almost all functions of the adaptive immune response. The primary mediator of T cell activation is the T cell receptor (TCR). The TCR is generated by recombination of genomic DNA sequences during T cell development in the thymus, and each TCR is essentially unique and is responsible for the specificity of each T cell [2]. Successful recombination of a functional TCR and emergence from the thymus results in a resting “naïve” T cell capable of migrating through the secondary lymphoid tissues and PB circulation, but yet incapable of producing any kind of immune response. Producing a T cell that is capable of mediating immune protection requires the activation of the naïve T cell via coordinated interactions between a number of molecules present on the T cell and the antigen-presenting cell (APC). APCs are cells that bear an antigenic peptide derived from the infectious agent bound to a major histocompatibility complex (MCH) class I or class II molecule. The most important APCs are the highly specialized dendritic cells, who ingest the antigen at the sites of infection and migrate to secondary lymphoid organs, where they present the antigen to T cells[3]. A part from dendritic cells, other cell types, such as macrophages and B cells can also act as professional APCs, although they are less powerful than dendritic cells at activating naïve T cells.

1.1.1 TCR formation and selection

TCRs are highly diverse heterodimers, consisting of a combination of α and β chains (αβ TCR) expressed by the majority of T cells, or γδ chains (γδ TCR) expressed in a small fraction of T cells in PB (1-5%) and T cells from mucosal sites[4]. The variable region of TCRα and δ chains is encoded by a number of variable (V) and joining (J) genes, while TCR β and γ chains are additionally encoded by diversity (D) genes[5,6]. During VDJ recombination, one random allele of each gene segment is recombined with the others to form a functional variable region (Figure 1.1). Recombination of the variable region with a constant gene segment results in a functional TCR chain transcript. Moreover, random nucleotides are added and/or deleted at the junction sites between the gene segments. This process leads to strong combinatorial and junctional diversity, resulting in a large and variable TCR repertoire, which will ensure the identification of a plethora of antigens.

Each TCR chain contains three hypervariable loops in its structure, termed complementary determining regions (CDR1-3). CDR3 region is encoded by the junctional region between the V and J or D and J regions, being therefore highly variable. It plays an essential role in the interaction of the TCR with the peptide-MHC complex, as it is the region of the TCR in direct contact with the peptide antigen. For this reason, CDR3 is often used as the region of interest to determine T cell clonotypes, as it is very unlikely that two T cells will express the same CDR3 nucleotide sequence, unless they have derived from the same clonally expanded T cell[6,7].

The sum of all TCRs by the T cells of one individual is termed the TCR repertoire. In T cells, the protective immune response relies on the presence of a T cell population that is poised to respond to peptides derived from pathogens, bound to self-MHC molecules. As the organism cannot predict the precise pathogen-derived antigens that will be encountered, the immune system relies on the generation and maintenance of a diverse T cell receptor (TCR) repertoire. Therefore, the size and diversity of the available T cell repertoire are crucial in shaping the immune response to a given antigen. The maximal theoretical diversity of TCRβ chain´s amino acid sequences in humans is estimated between 5 x 10^{11} [8] and 10^{14} [9]. However, whole TCRβ chain repertoire size in a single human organism is estimated at 1-5 x 10^{6} [8,10,11].

The large diversity of TCR means that there will usually be at least a few that can bind to any given foreign antigen. However, as each lymphocyte has a different receptor, the numbers of lymphocytes that can bind and respond to any given antigen is very small. In order to generate sufficient antigen-specific effector lymphocytes to fight an infection, lymphocytes with appropriate receptor specificity must be activated to proliferate and differentiate into effector

cells. Lymphocyte activation and proliferation is initiated in the draining lymphoid tissues, where naïve lymphocytes and activated APCs come together. Here, antigens are presented to the naïve circulating lymphocytes which migrate through the lymphoid tissue before returning to the bloodstream.

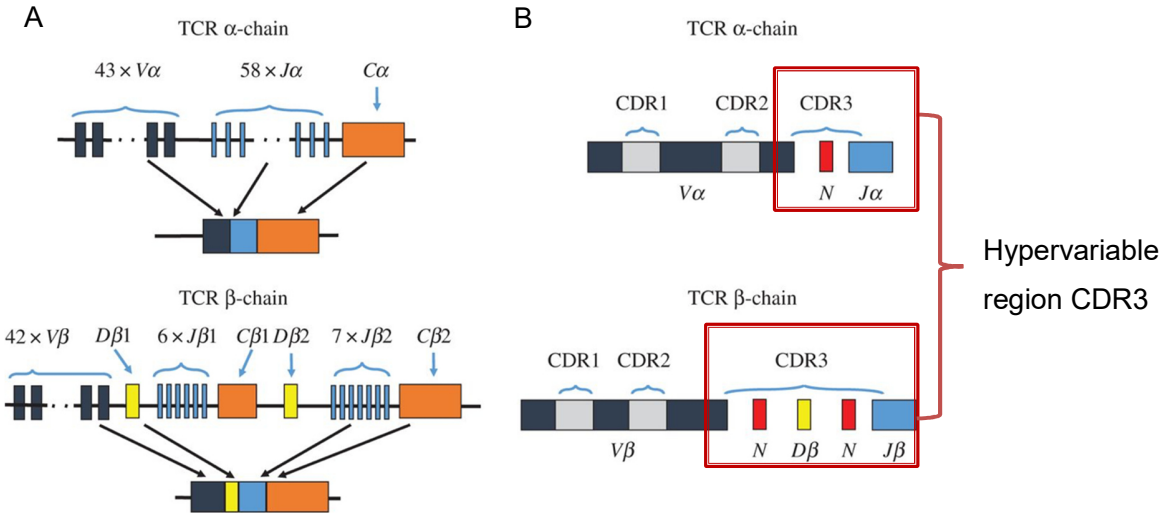


Figure 1-1: Gene rearrangements at the *TCR* loci. (A) Functional TCRs are heterodimers consisting of an α-chain and a β-chain that are generated by somatic gene recombination of variable (V), diversity (D) and junctional (J) gene segments for the β-chain, and V and J gene segments for the α-chain. During T-cell development, gene segments recombine and are spliced together with the constant region (C) to form the functional αβ TCR, with each T cell expressing only one type of recombined receptor complex. (B) The hypervariable complementary regions CDR1 and CDR2 are encoded on the V regions, while the most variable CDR3 region is created by the juxtaposition of different V (D) J germline segments after somatic recombination, with the diversity of the naïve TCR repertoire increased further by a lack of precision during V (D) J gene rearrangement and by the addition of non-template-encoded nucleotides (N) at the V (D) J junctions. Adapted from: Laydon DJ, Bangham CRM, Asquith B. 2015 Estimating T-cell repertoire diversity: limitations of classical estimators and a new approach.Phil. Trans. R. Soc. B 370: 20140291.

1.1.2 T cell lineages and differentiation

T cell support of immune responses comes into two categories: generation of “helper” $CD4^+$ T cells and generation of “cytotoxic” $CD8^+$ T cells. $CD4^+$ T cell responses support the immune system by the robust generation of cytokines and chemokines that either activate neighboring cells to perform specific functions (cytokines) or recruit new immune cell subsets to the site of pathogen encounter (chemokines).On the other hand, the function of $CD8^+$ T cells is largely

focused on the elimination of pathogen-infected cells by cytotoxic means, although they can also produce a diverse array of cytokines.

Due to the diverse spectrum of pathogens encountered (viruses, bacteria and parasites); the host produces an array of specialized T cells to fight the invading pathogen. Naïve T cells retain their specificity by the expression of their unique TCR but remain uncommitted to their helper fate until engagement of their TCR is accompanied by the integration of molecular signals downstream of their cytokine receptors. In response to the specific cytokine environment (cytokine milieu), antigen-stimulated T cells will be transcriptionally programmed into a variety of potential subsets that possess effector mechanisms appropriate for eliminating the pathogen. Helper T cell responses are thus classified into multiple T helper (Th) subsets, with the major ones designated as Th1, Th2, Th17, Th9,T follicular helper (Tfh) and T regulatory (Tregs)[12].

Interferon (IFN)-α/β and interleukin 12 (IL-12) generated in response to intracellular pathogens stimulate responding T cells to induce the expression of the transcription factor T-bet[13], promoting their differentiation into the Th1 subset[14]. The Th1 subset mediates defense against intracellular pathogens, and is characterized by the major production of IFNγ and TNFα[12,15,16].

Th2 cells are characterized by the production of IL-4, IL-5 and IL-13[15], and are generated after activation in the presence of IL-4 produced by a variety of innate cell types in response to parasites[17]. Cytokines produced by Th2 cells activate neighboring eosinophils, mast cells and basophils, which are specialized in the elimination of parasites[18]. Interactions with other parasites that generate milieu rich in IL-4 and transforming growth factor (TGF)-β result in the generation of the Th9 cell subset[19,20], which mainly produces IL-9[21]. Both Th2 and Th9 subsets up-regulate the transcription factor GATA-3[22], which results in their unique helper characteristics.

In response to extracellular bacteria and fungi[23], innate immune cells generate high amounts of TGF-β and IL-6[24], which together with the presence of IL-21 and IL-23 stimulation results in the generation of Th17 helper cells[25]. Those cells are regulated by the retinoic-acid-receptor-related orphan nuclear receptor gamma (RORγT)[26], and mainly produce IL-17 and IL-21[27]. They are important in activating neutrophils and recruiting them to the site of fungal and bacterial invasion[28].

Even though the inflammatory environment is strongly influenced by the specific nature of the invading pathogen, not all T cell differentiation is pathogen-specific. Some T cell differentiation occurs to support immune functions common to all infection responses. For instance, IL-21 and IL-27 stimulation are generated in response to a variety of pathogens and serve to polarize naïve T cells to Tfh subset, via the induction of B-cell lymphoma 6 protein (Bcl-6)[29]. Those cells

specifically home to B cell follicles in secondary lymphoid organs and assist in germinal center reaction of B cells, promoting the robust generation of high-affinity antibodies[30].

Naïve CD4⁺ T cells can also differentiate into suppressive Treg cells. These cells produce IL-10 and/or TGF-β, and their differentiation and function is driven by the expression of forkhead helix transcription factor (FOXP3)[31].The effects of Treg cells are the suppression of T cell proliferation and cytokine production from T cells via different mechanisms, such as production of IL-10[32].

Similarly to CD4⁺ T cell lineages, Type 1, 2 and 17 cytotoxic CD8⁺ T cells have been identified (Tc1, Tc2 and Tc17), and are also generated depending on the cytokine environment[33].

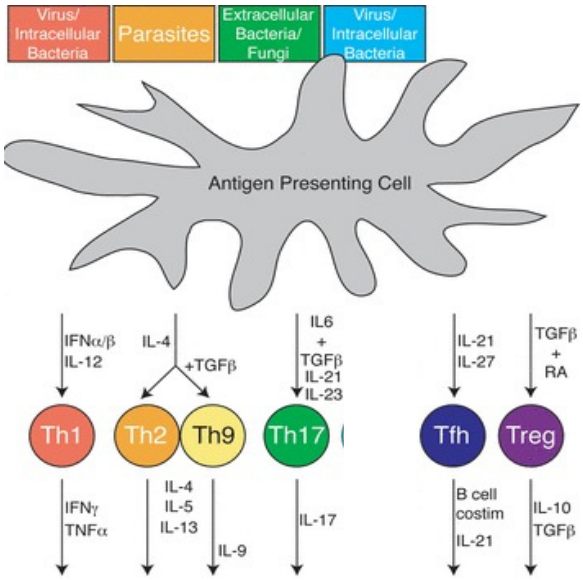


Figure 1-2: Together with TCR signaling, milieu cytokines promote differentiation of naïve T helper cells to one of a variety T cell subsets, programmed by transcription factors to specifically respond to the spectrum of pathogens. Upon differentiation, T cells themselves produce cytokines, which feed back into the cellular milieu, amplifying and balancing the immune response to promote specific pathogen clearance. Adapted from Nathan P. N. et al., (2013). T cell responses: Naïve to memory and everything in between. Adv Physiol Educ 37: 273–283

1.2 Immunological memory

The concept of immunological memory refers to the capacity of the immune system to "remember" previously encountered antigens and mount accelerated and enhanced response upon secondary re-challenges.

Already in 1781, in the remote Faroe Islands, a measles outbreak provided an insight into the mechanism of long-term protective immunity against infectious diseases. After this first outbreak, the Faroes remained measles-free for 65 years, until a major outbreak in 1846 that affected 75-95% of the population. The Danish physician Ludwig Panum made the observation that none of the elderly people who were infected with measles in 1781 had the disease for the second

time. Moreover, he also noticed that all the elderly people who had not suffered from measles in earlier life were attacked when they were re-exposed to infection. Panum´s study made two points: first, that immunity to measles was long-lived and second, that re-exposure to the virus was not essential for maintaining this long-term protective immunity[34].

In vaccinia virus-specific responses in mice, the kinetics and duration of T cell responses showed strong $CD8^+$ responses at day 7 post-infection, followed by a decline and stabilization at day 30 until day 300 after infection. Vaccinia-specific $CD4^+$ T cell responses in mice also peaked at 1 week after infection, reaching their maximum from 1 to 7 months after initial exposure [35]. Moreover, vaccinia-specific memory B cells can make up to 1% of circulating IgG memory B cells from 1 to 6 months after vaccination, followed by a decline of ~90% after the first year and a stabilization, being vaccinia-specific memory B cells maintained for more than 50 years [36]. Studies performed in humans also demonstrated the persistence of immune memory. For example, antigen specific $CD4^+$ and $CD8^+$ T cells can be identified 60 years following vaccination against smallpox, even when the virus was already eliminated and antigenic re-encounter was excluded [37], and immunity to smallpox vaccination has been proved to last at least for 35 years[38]. Moreover, studies performed using measles vaccination showed that even decades after acute measles and in the absence of demonstrable persisting virus, both $CD4^+$ and $CD8^+$ T cell pools still contain measurable levels of measles-specific memory T cells[39]. These data supports the idea that immune memory is long-lasting.

Several studies have shown that the induction of memory T cells, memory B cells and long-lived plasma cells are major components of the success of vaccines and protection against re-infection with previously encountered pathogens[40,41]. Plasma cells provide protection by constant antibody production, whereas memory B and T cells are endowed with unique properties that allow more vigorous and specific responses upon reinfection[42].

1.2.1 Generation of memory T cells

Memory B cells, plasma cells, and memory T cells play central roles in immunological memory but to date, the pathways that give rise to memory cells and how are they maintained for such long-periods remains poorly understood.

The generation of memory T cells is usually divided in three different phases. The initial one, defined as an activation phase, is characterized by the expansion of antigen-reactive T cells. In this phase, antigen-specific T lymphocytes undergo a clonal expansion, reaching an increase in numbers up to 5000-fold. This massive T cell proliferation is critical to long-term immunity

because the magnitude of the initial clonal burst typically determines memory T cell numbers [43]. Moreover, they acquire cytotoxic activity, up-regulate the secretion of effector cytokines and/or express peripheral tissue homing receptors, which will allow them to be recruited to the site of infection. Effector T cells migrate to the sites of infection and eliminate the pathogen by killing infected cells, producing cytokines, and/or recruiting other leukocytes via chemokine production.

Once the antigen has been cleared, sustained effector function could result in harmful immune inflammation. For this reason, most effector cells die during a second phase of contraction, characterized by a rapid decline in the frequencies of antigen-specific T cells that die by apoptosis via activation-induced cell death [44] or by neglect due to growth factor withdrawal [45]. A small proportion of antigen-specific T cell survives to this phase of contraction and constitutes a pool of memory T cells, maintaining then T cell oligoclonal specificities that were successful in controlling dissemination of the specific pathogen. Finally, in the third phase called the memory phase, antigen-specific T cells that survived the phase of contraction (typically 5-20%) develop into specialized memory T cells, generating a long-lived and stable pool of memory T cells [46,47]. Memory T cells persist in an antigen-independent, but apparently cytokine dependent manner[48].

Different models have been developed to study the generation of memory T cells after antigen encounter. The first model, known as linear model, suggests that naïve $CD4^+$ T cells, after activation by cognate antigen-MHC complexes, differentiate into effector cells and then into memory cells[49]. A linear differentiation pathway was also proposed for $CD8^+$ T cells[50]. A second model, known as divergent model, proposes that memory T cells are directly generated from naïve T cells after antigen recognition, without going through the effector stage[51]. Recently, an asymmetric division model was proposed, in which effector and memory cells are formed simultaneously upon the first cell division of naïve progenitor T cells [52].

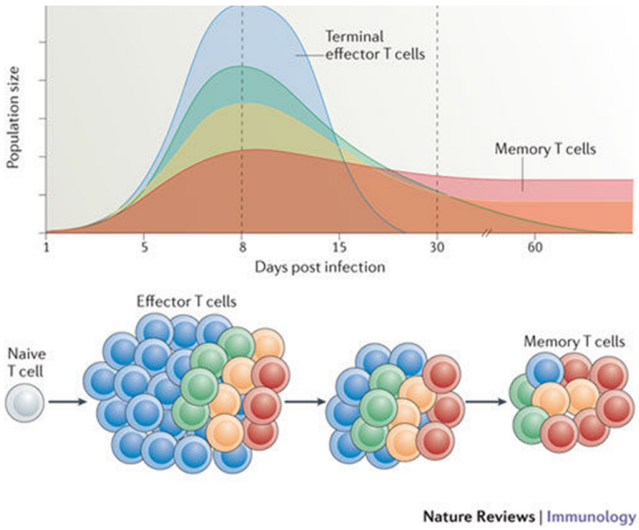


Figure 1-3: Formation of memory T cells following T cell responses. Upon infection, naïve T cells become activated and proliferate and differentiate into a heterogeneous population of effector T cells. Most of the effector T cells terminally differentiate into effector cells (blue cells) that protect against the current infection, but have no potential to develop into memory T cells. A smaller subset of effector T cells persists to develop into different types of memory T cells. Memory T cells form a potent defense system. Adapted from: Cui W and Kaesch SM., (2012). Transcriptional control of effector and memory CD8 T cell differentiation. Nat Rev Immunol 12:749-61

1.2.2 Functional characteristics of memory T cells

A central feature of the adaptive immune system is the capacity of memory T cells to mediate faster, stronger and more effective responses to secondary pathogen challenge than naïve T cells [53,54]. Memory T cells are thought to have an increased sensitivity to the antigen compared to primary responding cells [55–57], probably due to the selection of the repertoire for higher affinity TCR clones dominating secondary responses. The ability of memory T cells to respond to lower doses of antigens may also be due to alterations in the levels of TCR and/or downstream signaling molecules. Kumar and colleagues found that CD3 molecules were more likely to form distinct clusters on the cell surface of memory compared with naïve T cells, allowing more efficient TCR triggering [58].

Moreover, memory T cells are mainly polyfunctional in terms of cytokine secretion[59]. Numerous studies in the past have evaluated the frequencies of antiviral $CD4^+$ T cells producing different cytokines as a measure of the magnitude and the quality of specific responses, which are critical for the control of viral responses. In human studies analyzing $CD4^+$ specific responses to viral antigens, multiple-cytokine-producing cells are functionally superior to single-cytokine-producing cells, having a major content of cytokine per cell, and showing a higher expression of CD154, which provides a better co-stimulation to $CD8^+$ T cells and B cells [60].

1.2.3 Heterogeneity of circulating memory T cells in terms of function and location

Naïve T cells are characterized by the expression of the lymph node homing CC-receptor 7 (CCR7), reflecting their major residence in secondary lymphoid tissues. Memory T cells in humans are classically distinguished by the expression of the CD45RO isoform and the lack of expression of the CD45RA isoform. However, $CD54RO^{+}CD45RA^{-}$ T cells are now known to comprise heterogeneous populations of memory T cell subsets. In 1999, Sallusto, Lanzavecchia and colleagues described the heterogeneity in human memory T cells from PB by the expression of CCR7, effector functions and proliferative capacity [61]. Based on this, memory T cells can be divided into $CD45RA^{-}CCR7^{+}$ central memory T cells (T_{CM}) and $CD45RA^{-}CCR7^{-}$ effector memory T cells (T_{EM}). T_{CM} cells are characterized by their ability to traffic to lymphoid tissues, proliferate upon activation, provide help to antigen presenting cells (APCs) via CD154, and secrete high amounts of IL-2. On the other hand, T_{EM} can migrate to peripheral tissues and are able to produce more effector cytokines like IFNγ. Interestingly, T_{CM} cells have the capacity to differentiate into T_{EM} cells upon secondary stimulation, with immediate effector functions [61]. The existence of T_{CM} and T_{EM} cell subsets in lymphoid and peripheral tissues was also confirmed in mouse models [62,63].

Most recently, additional memory cell subgroups have been described, suggesting further heterogeneity of memory T cells. Memory stem cells (T_{SCM}) are defined by the expression of naïve ($CD45RA^{+}$ and $CCR7^{+}$) and memory ($CXCR3^{+}$ and $CD95^{+}$) markers, and represent the most stable memory subset [64–66]. Transcriptional analysis of human $CD4^{+}$ T cell populations positioned T_{SCM} cells as a distinct population with a transcriptional profile between naïve and T_{CM} cells [67]. Memory stem T cells have also been described in non-human primates, where they were found in PB, secondary lymphoid organs and the BM [68].

1.3 Tissue resident memory T cells (T_{RM})

In addition to circulating memory T cells, mouse studies have also established the existence of a non-circulating T_{RM} subset as a non-circulating subset that resides in peripheral tissue sites and elicits rapid in situ protective responses[69]. Memory T lymphocytes persisting in different tissues have attracted increasing interest because their considerable contribution to the long-live memory T cell pool[70]. However, limited access to human tissue samples and the limitations of

current methods to dissociate memory T cells in peripheral organs, limits our current understanding on the presence and activities of these cells[71].

In humans, only a small proportion of T cells can be found in blood circulation (5-10 x 10^9 cells), in contrast with other tissues, where the T cell numbers are much higher. Estimates of T cell numbers in human tissues are 2 x 10^{10} in the skin [72,73], 1 x 10^{10} in the lungs [74], 3 x 10^{10} in the gut [75], 15 x 10^{10} in lymph nodes, 3 x 10^{10} in spleen, and 2,5 x 10^{10} in BM [76]. These observations demonstrate that both lymphoid and non-lymphoid tissues are important when investigating memory T cell formation and maintenance. In particular, several studies have demonstrated the presence of T_{RM} cells in a variety of tissues, such as BM, gut, skin, lungs, brain and thymus [59,62,72,74,77–82]. Moreover, animal studies performed in skin and vaginal mucosa indicated that T cell-mediated memory responses are highly compartmentalized in tissue sites, and that T_{RM} cells mediate responses with a higher protective capacity compared to circulating T_{CM} cells[79,82–84].

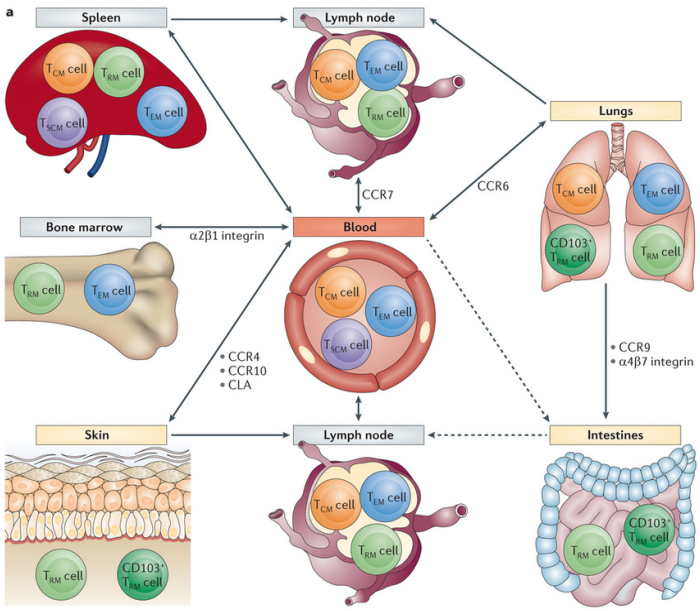


Figure 1-4: Schematic view of blood and tissue compartmentalization of memory T cells. The distribution of different memory T cell populations is shown in the figure, including central memory T cells (T_{CM}), effector memory T cells (T_{EM}), stem cell memory T cells (T_{SCM}) and tissue resident memory T cells (T_{RM}). Distinct populations of memory T cells have the capabilities to circulate through the blood (red), lymphoid organs (grey) or peripheral tissues (yellow). Figure modified from: Donna L Farber et al., Human memory T cells: Generation, compartmentalization and homeostasis. Nature Reviews Immunology. 2013; doi:10.1038/nri3567.

1.3.1 Generation of T_{RM} cells

During the acute phase of an infection, recently activated effector T cells enter the inflamed tissue in order to clear the pathogen. Heterogeneous populations of effector T cells have different potential to become memory T cells depending on the expression of transcription factors and cell surface molecules. Moreover, external factors like T cell receptor (TCR) signal strength, and the presence of inflammatory cytokines can also influence the effector and memory T cell differentiation and fate[85,86]. The molecular mechanisms required to control T_{RM} differentiation are still poorly understood. It is possible that T_{RM} cell development involves several checkpoints, such as tissue entrance, local retention and T_{RM} formation and survival.

The ability of effector T cells to migrate into non-lymphoid tissues, may be a key for subsequent T_{RM} cell differentiation. T cells that lack CCR7 expression show enhanced local conversion to the T_{RM} cell phenotype in the skin[87]. Moreover, CD69-deficient $CD8^+$ T cells show defective T_{RM} cell development in the skin [88] and the lungs [89] and BM[90], suggesting that CD69 is a critical tissue retention marker.

The cytokine transforming growth factor-β (TGFβ) was shown to have a role in the development of $CD69^+CD103^+$ T_{RM} cells in the skin, gut and lungs [87,91,92]. Moreover, the cytokines interleukin-33 (IL-33) and tumor necrosis factor (TNF) in combination with TGFβ can induce a T_{RM} like phenotype ($CD69^+CD103^+$) [93]. The homeostatic cytokine IL-15 may also be a decisive factor in T_{RM} differentiation and survival. For example, in the absence of IL-15, $CD8^+$ T_{RM} cells do not persist in the skin after HSV infection [87].

1.3.2 Phenotype and migratory properties of T_{RM} cells

The majority of T_{RM} cells in lymphoid and non-lymphoid tissues express two surface cell markers: the trans membrane C-type lectin CD69 and the integrin α-E CD103. CD103 can bind to E-cadherin[94], which may have a role in the retention of T lymphocytes into tissues. Indeed, $CD8^+$ T cells genetically deficient in CD103 are able to migrate to the small intestine epithelium, brain and skin epidermis, but are not retained [81,87,93]. However, some evidence indicates that the majority of putative T_{RM} cells in many tissues do not express CD103 [69,93], suggesting that it is very likely that many T_{RM} cells do not depend, or do not even express CD103. Other integrins that bind extracellular matrix proteins in the lamina propria or basement membrane might also retain T_{RM} cells in different tissues. For instance, the integrin α1β1 (VLA-1) binds laminins and collagens and is highly expressed on $CD8^+$ T_{RM} cells from the brain, small intestine, lung and skin[79,87,95,96].

In addition, T_{RM} cells have elevated expression of the C-type lectin CD69. Functionally, CD69 has been shown to induce G-protein coupled sphingosine-1-phosphate receptor1 (S1PR1) down-regulation. S1PR1 mediates T cell egress from lymph nodes by inducing chemotaxis to sphingosine-1-phosphate (S1P) present in efferent lymph [97]. Thus, inhibiting S1P responsiveness might represent an important checkpoint for the generation of T_{RM}. CD69 is induced on T_{RM} after they migrate to the sites of residence [87,98,99]. However, it remains to be determined whether all T_{RM} require CD69, or whether other means of S1PR1 inhibition are sufficient for T_{RM} maintenance. Notably, transcriptional regulation of S1PR1 is driven by the Kruppel-like factor 2 (KLF2). TGF β, IL-33 and TNF present in the tissues induce down-regulation of KLF2 expression and consequently suppress S1PR1 expression [99]. Interestingly, KLF2 was also shown to directly promote the expression of CD62L[100] and had increased correlation to CD69 expression[99]. Therefore, the down-regulation of KLF2 and S1PR1 and the up-regulation of CD69 might both play functional roles in the development and retention of T_{RM} cells.

Two studies performed in humans examined naïve and memory T cell subsets from several tissues obtained from organ donors, showing that substantial numbers of both $CD4^+$ and $CD8^+$ memory T cells in spleen, lymph nodes, lungs and intestines expressed the marker CD69 [101,102]. Moreover, the expression of CD69 has also been detected in memory T cells from human BM [59].

To conclude, CD103 and CD69 seem to be important for the generation and maintenance of T_{RM} cells, but there might be other factors that promote residence and recirculation.

1.3.3 Maintenance and functional capacity of T_{RM} cells

An important feature of T_{RM} cell populations in non-lymphoid tissues is their long-term persistence. In mice, T_{RM} cell populations can remain numerically stable in skin for several months[79,82,83]. Moreover, studies performed in humans have shown the existence of memory $CD4^+$ T cells specific for childhood antigens in BM of elderly patients, even though when they were no longer detectable in blood circulation [59]. Until now, it is not clear whether T_{RM} cell populations can be replenished in the steady state from recirculating memory T cells or whether and how they are maintained as a stable population. More experiments need to be performed in order to address the question about T_{RM} persistence. As T_{RM} cells in lymphoid organs do not recirculate, they have limited access to blood-derived signals, so the local availability of nutrients may also influence T_{RM} cell maintenance and functions. Moreover, as the space in different tissues may limit the number of T cells that persist locally, there is the possibility of competition

between T_{RM} cell populations of different specificities. Such competition does not seem to affect circulating memory T cells, as populations of T_{EM} can increase in size after immunization without affecting the naïve T cell pool [103]. IL-15, TGFβ, TNFα and IL-33 have all been implicated in the generation and maintenance of T_{RM} cells [87,99]. TGFβ, TNF and IL-33 have been shown to have a role in induction of CD103 expression and acquisition of a T_{RM} phenotype, although their role in maintaining these cells in specific tissues for long-time periods has still to be proven.

Upon re-exposure to a pathogen, circulating memory T cells (mostly T_{CM}) are re-stimulated in draining lymph nodes where they proliferate and generate large numbers of effector T cells, which will migrate to infected tissues after several days. On the other hand, circulating T_{EM} cells can be recruited directly to the inflamed tissue within hours or days, providing a faster response. Taking this into consideration, circulating memory T cells may not offer the most effective local protective immunity in peripheral sites [82,83,104,105]. Moreover, there is emerging evidence that T_{RM} cells can be multifunctional and that they have qualitative functional differences. For example, human BM T_{RM} cells are polyfunctional in terms of cytokine production [86,105], and a great number of human lung T_{RM} [106] and intestinal T_{RM} cells produce multiple pro-inflammatory cytokines. Recent studies have shown that T_{RM} cells can function in recall responses through effector mechanisms other than killing. For example, they can respond to antigens *in situ* by producing different cytokines such as IFNγ, which enhance the recruitment of circulating T cells from the blood [107,108].

In humans, antigen-specific memory T cells are generated and dynamically maintained as a heterogeneous T cell population in the context of thousands of different pathogens that are introduced at various stages of life. Several studies have shown that there is a higher generation and maintenance of virus-specific effector/memory T cells in tissues compared with circulation. For instance, cutaneous challenge with varicella zoster virus resulted in memory T cells accumulation in the skin [109]. In addition, lung tissue contained an increased frequency of influenza virus-specific memory $CD8^+$ T cells compared with blood [110,111] and human BM was found to be also enriched with measles, mumps and rubella antigen-specific $CD4^+$ memory T cells compared with blood [59]. These findings suggest that there is a compartmentalization of pathogen-specific memory T cells in different tissues.

1.4 BM memory T cells

The BM is a primary lymphoid tissue, were major part of hematopoiesis occurs. It is formed by islets of hematopoietic-active regions and fatty areas, which mainly contain adipose tissue[112], all contained inside central cavities of long bones. In addition to its hematopoietic function, the BM plays a role in several physiological and pathological processes, including B and T cell memory, long-term antibody production, inflammatory response, bone metabolism and tissue repair [42,59,113–117].

In both humans and mice, mature T cells represent 3-8% of total nucleated cells in the BM [118,119]. BM $CD4^+$ and $CD8^+$ T cell populations contain a high proportion of cells with a memory phenotype, expressing low levels of CD45RA in humans [59], and high levels of CD44 in mice[118]. Several studies have pointed out that, long time after priming, memory T cells can be found all over the body[120], with a preferential enrichment either in the BM[59] or at the site of pathogen entry[109–111], even in the absence of residual antigen. BM resident memory T cells are then thought to contribute to systemic memory, whereas T_{RM} cells in peripheral tissues participate in local protection.

1.4.1 Regulation of T cell migration and retention into the BM

In the BM, there is no lymphatic drainage present, so BM exchanges with the rest of the body only occurs via blood circulation. Indeed, the BM is the only lymphoid organ which is not integrated in the lymphatic system. T lymphocytes can enter the BM only by crossing flat endothelium-lined vessels. T cells attach to the endothelium through selectin- and integrin-mediated interactions, which allow T cell migration across the vascular endothelium [121]. After that, firm adhesion is mostly mediated by interaction of the lymphocyte integrin α4β1 (VLA-4), expressed at high levels by activated T cells [122], and the endothelial adhesion molecule VCAM-1, which is constitutively expressed by BM microvasculature and stromal cells[123]. CXCL12 (stromal cell-derived factor-1 SDF-1) has been shown to be important in cell attraction to the BM, in addition to hyaluronic acid[124]. This chemokine is expressed at high levels by sinusoidal endothelium in the BM and it is recognized by the chemokine receptor CXCR4, which is expressed by T cells.

Molecular regulation of T cell egress from the BM involves Sphingosine-1-phosphate (S1P) interaction with its receptor S1P1[125]. Bankovich and colleagues demonstrated the biochemical interaction between CD69 and S1PR1 by western blot [126], and then, it was suggested that

binding of CD69 to S1PR1 initiates its internalization and degradation[127]. In concordance with these findings, it was observed that $CD4^+$ memory T cells accumulated less in CD69 ko mice compared with the WT counterparts, so it was proposed that CD69 could mediate retention of memory $CD4^+$ T cells in the BM [128]. Relocation of antigen-specific memory $CD4^+$ T cells to the BM has been suggested to be in a CD69 and Integrin-α2 dependent manner, as cells lacking the expression of one of those molecules failed to immigrate into BM[128,129].

1.4.2 Functional long-term memory provided by BM memory T cells

Several studies have shown that the contribution of BM memory T cells to systemic immunity is greater than previously thought [59,123,130]. For example, it has been long known, that T-B cell cooperation in the BM can lead to antibody formation[113]. Moreover, BM memory $CD4^+$ T cells were shown to be polyfunctional, expressing multiple effector cytokines, a property that has been correlated with enhanced immunological protection [59].

The human BM T cell population is significantly enriched for specificities against systemic pathogens, which represent persistent, recurrent and childhood immunological challenges. Pathogen-specific memory T cells specific for acute viral infections, such as measles, mumps and rubella (MMR) [59], and also persistent viruses, such as cytomegalovirus (CMV), Epstein-Barr Virus (EBV), and Human Hepatitis C virus (HCV) [131–133]could be detected in the BM. However, when looking at typical skin or mucosa pathogens such as vaccinia virus or Candida albicans-MP-65, $CD4^+$ T cells specific for these antigens are not enriched in the BM, suggesting that they may be mostly maintained locally as T_{RM} in skin and mucosal sites[59]. Of note, MMR-specific memory $CD4^+$ T cells were shown to be maintained in the BM of elderly individuals, even when they are non-detectable in blood circulation[59], indicating that BM is the site for long-term maintenance of memory T cells specific for systemic antigens. In some cases, antigens were expressed in the BM, for example in the EVB infected subjects [132]. However, the presence of antigen in the BM was not reasonable in other cases, for example long time after vaccination against tetanus or other childhood antigens[59,134]. Therefore, it appears that long-lived antigen-specific memory T cells go to the BM during immune responses to a high variety of antigens, and are maintained there without the presence of the antigen.

1.4.3 Long-term survival of BM memory T cells

It was shown that memory T cells in the BM are resting in terms of activation, proliferation and transcription [59,135]. The memory T cell pool acts as a repository of heterogeneous long-lived T cells that had been previously in contact with the antigen. To date, no unique mediator has been described as an inducer of memory T cell survival. It is possible that multiple signals with redundant or partially overlapping functions are involved to ensure the optimal survival of memory T cells. The anatomical location of the niches where T cells localize in the BM, and the cellular components of these niches are still under investigation. One major problem when investigating the "BM stromal niches" is the high degree of stromal cell heterogeneity. For example, it was shown that BM stromal cells expressing high levels of CXCL12 were different from those providing IL-7 [136]. Each type of stromal cells was associated with B lymphocytes at different developmental stages, and it could be seen as a mechanism to maintain immunological memory of different cell types with different requirements preventing competition between them.

1.4.3.1 Relationship of BM memory T cells with the local microenvironment

Hormones, antigen receptors, regulatory cells and, in particular, cytokines are the principal signals for maintaining lymphocyte homeostasis[137]. In the BM, reticular-like BM stromal cells have been shown to produce high levels of IL-7, IL-6 and VCAM-1 [138–141], which are involved in the maintenance of B lymphocytes, long-lived plasma cells and T cells.

Histological analysis of memory $CD4^+$ T cells in the murine BM revealed direct contact of these cells with $VCAM^+$ IL-7 producing stroma[77]. Also, murine $CD8^+$ memory T cells were found to reside in close proximity with IL-7^+ stromal cells[135], and clusters of memory T cells and IL-15 producing cells were observed in human BM sections [78]. Dendritic cells, monocytes, $CD34^+$ hematopoietic cells and BM stromal cells have been described to produce IL-15 [142,143].

Several studies have highlighted the role of cytokines that signal through the common γ-chain receptor (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21) in the survival and homeostatic proliferation of lymphocyte populations [137,144]. Especially, several groups have demonstrated the involvement of IL-7 and IL-15 in the survival of memory $CD8^+$ T cells [145–147], whereas their involvement in the persistence of $CD4^+$ T cells remains more controversial [144,148]. The receptor for IL-15, assessed by the expression of the β-chain (CD122), is expressed at significantly higher levels on memory $CD8^+$ T cells compared to memory $CD4^+$ T cells [149]. This could explain why IL-15 has been shown to have an important role on the homeostasis of memory $CD8^+$ T cells but not so much in

memory $CD4^+$ T cells [144,149–151]. In contrast, the receptor of IL-7, measured as CD127 expression is expressed at comparable high levels on both populations of memory T cells [152].

Apart from the soluble factors present in the BM survival niches, cell-cell contacts generated between T cells and stromal cells are also thought to be important for the maintenance of memory T cells. BM resident memory T cells express both, VLA-2 and VLA-4, the alpha2- and alpha4-beta1-integrin heterodimers, which bind mainly to collagens I, II and XI [153,154], and VCAM-1[155,156] respectively. The importance of adhesion signals presumably mediating contact to stromal cells has been demonstrated for several immune cell types. For example, the disruption of cell-cell contact between hematopoietic cells and stroma via VLA-4 and VCAM-1 interaction resulted in an increase of apoptosis of $CD34^+$ hematopoietic cells [157], and antibodies that block VLA-4 in plasma cells have been shown to eliminate these cells from the BM [158]. Regarding VLA-2, the homing of adoptively transferred T cells to BM can be blocked by antibodies against this integrin [77]. Moreover, collagen XI, one of the putative ligands for VLA-2 is exclusively expressed in the BM [129], although its implication in memory T cell maintenance remains to be determined.

Other factors that could influence the potential survival of BM memory T cells compared with the PB circulating ones are the levels of oxygen. Oxygen supply and diffusion into tissues are necessary for survival. The oxygen partial pressure (pO_2) results from the balance between oxygen delivery and its consumption. In mammals, oxygen is transported by red blood cells circulating in a well-organized vasculature. In a physiologycal condition, each organ and tissue are characterized by their own unique “tissue normoxia”. In some human studies, it has been estimated that arterial blood has a pO_2 = 13.2, whereas this is reduced in venous blood (pO_2 = 5.3) and in BM (pO_2 = 6.4±0.6) [159]. Taking that into account, BM resident memory T cells may present an adapted metabolism to the local environment, which favors their survival in reduced oxygen conditions.

Long-term memory T cell maintenance may not be simply influenced by single factors, but rather via the combination of different conditions present in the BM survival niches, which at the end will ensure the long-term survival of resting memory T cells.

1.4.3.2 Molecular mechanisms involved in the maintenance of memory T cells

The JAK/STAT, PI3K/AKT and MAPK/ERK signaling molecules are major cell survival pathways. Signals transduced through cytokines or other molecules and their specific receptors can activate these pathways that promote survival and inhibit cell death. Cytokine signaling through

tyrosine kinases can lead to the transcription of anti-apoptotic factors, but the exact molecular mechanisms implicated in memory T cell survival are still under investigation.

The members of the B-cell lymphoma 2 (BCL-2) family can be assigned to three groups. The pro-survival members are BCL-2, BCL-X_L (also known as BCL-2L1), BCL-W (also known as BCL-2L2), MCL1 (myeloid cell leukemia sequence 1), A1 and BOO (also known as (BCL-2L10). Pro-survival factors prevent the effectors BCL-2-associated X protein (BAX) and BCL-2 antagonist/killer (BAK), which are considered the second group, from inducing mitochondrial outer membrane permeabilization, thereby preventing the activation of caspases [160,161]. The third group includes the BCL-2-homology domain 3-only proteins, BIM, BAD (BCL-2-antagonist of cell death), BIK (BCL-2-interacting killer), BID (BH3-interacting-domain death agonist), harakiri (HKR), NOXA (NADPH oxidase activator 1), PUMA (p53-upregulated modulator of apoptosis; also known as BBC3) and BMF (BCL-2-modifying factor). Group three members sense the stress signals and release the inhibition imposed on BAX / BAK by the pro-survival proteins [162].

When BH3-only proteins are induced or activated, they interact with BCL-2 family proteins to promote apoptosis. Various studies indicate that some BH3-only proteins, such as BIM and PUMA, bind to all anti-apoptotic BCL-2 family members, whereas others, like BAD and NOXA bind only certain anti-apoptotic BCL-2 family members, which are BCL-2, BCL-XL and BCL-W for BID and MCL-1 and A1 for NOXA [163–165]. Interaction between the pro- and anti-apoptotic proteins prevents the inhibition of BAX and BAK by the pro-survival molecules and promotes caspase activation by its interaction with mitochondria, which will lead to apoptosis.

Regulation of the expression levels of anti-apoptotic BCL-2 family proteins controls apoptosis. For example, BCL-2 and BCL-XL can be transcriptionally induced by growth factors through the Janus-kinase-signal transducer and activator of transcription (JAK/STAT) pathway to promote cell survival [166]. Moreover, MCL-1 is rapidly degraded by the ubiquitin-proteasome pathway in response to cytokine deprivation or other death stimuli, and can be up-regulated post-transcriptionally to prevent apoptosis by inhibiting the rate of degradation [167]. NOXA is expressed at low levels in different tissues [168] and its expression is induced as a response for DNA damage [168], cytokine signaling [169] and hypoxic conditions [170]. On the other hand, regulation of expression of the pro-apoptotic proteins BAX and BAK is less apparent and they appear to be constitutively expressed at constant levels, so these proteins are mainly post-translational regulated by other members of the BCL-2 family.

BIM has shown to be important for memory T cell maintenance. In $Bim^{-/-}$ mice, resting memory $CD8^+$ T cells accumulated in the periphery [171], suggesting its implication in memory T cell survival. BCL-2 and MCL-1 have both a shared role, the inhibition of BIM. Both are required for

the survival of developing thymocytes and mature T cells [172,173]. Studies from mice, primary cells and cell lines have implicated the BCL-2 family, especially the BIM/BCL-2 axis downstream γ-chain cytokine signaling [137], but the role of MCL-1 in cytokine-induced survival is less well described. In addition, studies performed in mice showed that IL-7 and IL-15 promote survival by up-regulating both MCL-1 and BCL-2. Each of the pro-survival molecules follow different pathways in promoting cell survival, so the balance between the functions of BCL-2 and MCL-1 may be important in regulating memory T cell survival [174].

NOXA selectively binds and targets MCL-1 for proteasomal degradation, event that has been considered to be a prerequisite for cell death in response to UV irradiation [175] and cytokine deprivation[173]. It has been shown the involvement of NOXA in the maintenance of memory Th1/Th2 homeostasis, but not in proliferating T cells [176].

The receptors for the two cytokines known to be important for T cell homeostasis, IL-7 and IL-15, share the common cytokine-receptor γ-chain, which is associated with Janus Kinase 3 (JAK3), a kinase that is likely to be involved in lymphocyte homeostasis [137]. Studies using mouse naïve T cells indicated that IL-7 stimulation is connected to the JAK1-3/STAT5 and the PI3K/AKT cell signaling pathways [177–179], which are major cell survival pathways. However, in vitro studies performed in effector/memory $CD4^+$ T cells, using IL-7 stimulation and specific inhibitors for these pathways suggested that the IL-7 pro-survival function is mediated through the JAK/STAT pathway, independently of PI3K/AKT [180]. Interestingly, the PI3K/AKT pathway was shown to be also important in maintaining basal cell viability [180], maybe by modulating other pro- or anti-apoptotic molecules of the BCL-2 family.

Taking together, a balanced relation between anti-apoptotic molecules and BH3-only pro-apoptotic molecules may play an essential role in preventing apoptosis and maintaining cell survival. Until now, it is not completely known which pathways are more important in the maintenance of memory T cells, but it is possible that different survival factors provided by the niches function through different signaling molecules, and the combination of all of them is the responsible for long-term memory T cell maintenance.

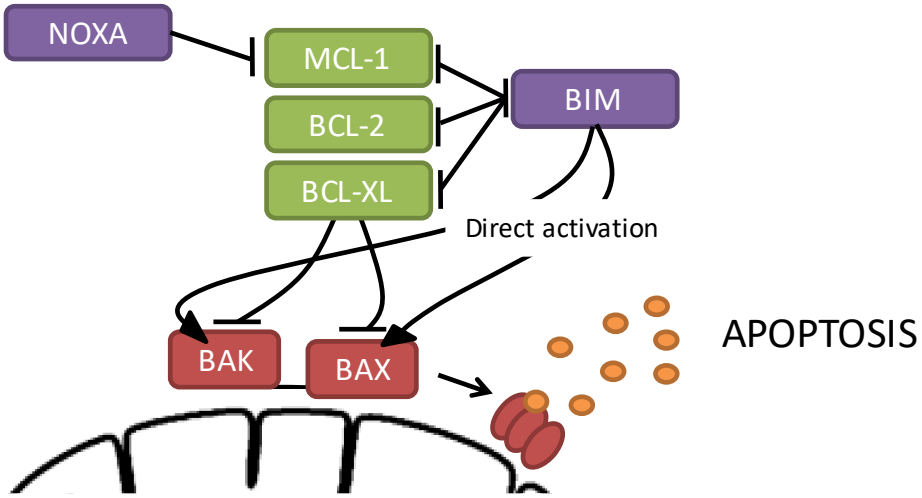


Figure 1-5: The BCL-2 family control of cell apoptosis. Pro-apoptotic BCL-2 family protein activation, such as BIM and NOXA, is triggered by extra- and intra-cellular signaling. De-repressor BH3-only proteins (NOXA) prevent or disrupt inhibition by MCL-1. Direct activator BH3-only proteins (BIM) bind BAK and BAX to induce their homo-oligomerization and cell death by apoptosis. Anti-apoptotic proteins (MCL-1, BCL-2 and BCL-XL) can interact with BIM to inhibit its interaction with BAX and BAK and suppress cell death by apoptosis.

1.5 Aim of the thesis and study objectives

With the discovery of T_{RM} cells, the previous concept that memory T cells are continuously circulating through the body has been challenged. T_{RM} cells can be found in a great variety of tissues, including the BM. Although T_{RM} cells are thought to confer us with higher local protection at the site of infection, the exact division of tasks and the differences on the lifestyle of resident versus circulating memory T cells remains poorly understood.

Previous work of our group showed that BM memory T cells are quiescently maintained for long times in specific survival niches, without signs of activation or circulation[59]. However, the survival signals as well as the survival mechanisms involved in this long-term maintenance of BM memory T cells remain poorly understood. In addition, memory $CD4^+$ T cells specific to systemic childhood antigens like measles, mumps or rubella (MMR) were enriched in the BM oh elderly donors, even when they were no longer detectable in blood circulation[59]. Whether both, the $CD69^+$ and the $CD69^-$ BM memory T cells maintain these long-term memory, and whether and how resident memory T cells specific for systemic antigens can be mobilized and reactivated to confer as with immune protections remains still unclear.

Therefore, in this thesis I hypothesized:

That BM memory T cells remain quiescent for long-periods of time in specific BM niches, where they receive diverse survival signals from their microenvironment. Due to the differences in the environment of resident and circulating memory T cells, both cell populations may require different factors and be maintained by different survival mechanisms.

That BM resident memory T cells form a pool of cells separated from the circulating memory T cells, which could be reflected by differences in their functional capacities and TCR repertoires.

That antigen-specific memory $CD4^+$ T cells to systemic antigens have the capability to be mobilized from their resting sites and mount a secondary immune reaction to confer us with faster and greater immune protection after systemic re-challenge.

I compared the survival mechanisms of resident BM $CD69^+/CD69^-$ with PB circulating $CD69^-$ memory $CD4^+/CD8^+$ T cells. I also investigated some of the essential factors for memory T cell maintenance as well as the molecular mechanisms triggered by them and involved in memory T cell survival. Furthermore, in order to determine the relationship between resident BM and circulating memory T cells, I compared the CDR3 TCRβ repertoires of BM $CD69^+/CD69^-$ and blood circulating $CD69^-$ memory $CD4^+/CD8^+$ T cells, as well as the capacity of both, the BM $CD69^+/CD69^-$ respond to antigen stimulation. Finally, in order to find out whether and how T_{RM} cells are able to react to systemic re-immunization and be mobilized into the blood to mount a secondary immune reaction, we I followed the dynamics of antigen specific memory $CD4^+$ T cells in response to a secondary MMR vaccination. Altogether, these results provide a better understanding of human T cell memory maintenance and functionality, helping us in the development of vaccines and immunotherapies.

2 Materials and methods

2.1 Materials

2.1.1 Reagents and kits

Reagent	Manufacturer	Remarks
10X BD FACS Lysing Solution	BD Biosciences	
10X BD FACS Perm Solution 2	BD Biosciences	
Anti-human CD4 Microbeads	Miltenyi Biotec	
Anti-human CD8 Microbeads	Miltenyi Biotec	
Brefeldin A	Biolegend	10 µg/mL
CD28 functional grade pur	Miltenyi Biotec	100 µg/mL
CD40 functional grade pur	Miltenyi Biotec	100 µg/mL
Cell Proliferation Dye eFluor 670	eBioscience	2,5 µg/mL
CFSE	Live Technologies GmbH	2,5 µg/mL
EDTA	Live Technologies GmbH	
EL Buffer	Qiagen	
FcR blocking reagent	Miltenyi Biotec	50µg/ml
Ficoll-Paque Plus	GE Healthcare	
Fetal Bovine Serum	Biowest	
Human FcR blocking Reagent	Miltenyi Biotec	
Human IL-6, premium grade	Miltenyi Biotec	1 µg/mL
Human IL-7, premium grade	Miltenyi Biotec	1 µg/mL
Human IL-15, premium grade	Miltenyi Biotec	1 µg/mL
Human IL-33, premium grade	Miltenyi Biotec	1 µg/mL
Human TGFβ, premium grade	Miltenyi Biotec	1 µg/mL
Human TNFα, premium grade	Miltenyi Biotec	1 µg/mL
Human male Ab serum	Sigma-Aldrich	Heat-inactivated
Inside Fix Solution	Miltenyi Biotec	
5X Lyse/Fix Solution	BD Biosciences	
MMR vaccine (Priorix)	GSK	For vaccination

Permeabilization Buffer A	Miltenyi Biotec	
Proleukin (IL2 clinical use)	Novartis	200 IU/mL
Propidium Iodide solution (PI)	Sigma-Aldrich	1 µg/mL
Pure Orange	In house	1 µg/mL
Q5 High-Fidelity DNA Polymerase	New England Biolabs	
Qiazol Reagent	Live Technologies GmbH	
RNAse inhibitor	Live Technologies GmbH	
RPMI Medium 1640 - GlutaMax	Gibco, Life Technologies	
Smartscribe Reverse Transcriptase	Clontech	
Trypsin-EDTA (1X)	Live Technologies GmbH	
Uracil-DNA glycosilase (UDG)	New England Biolabs	
X-VIVO 15 Chemically defined medium	Lonza	

Kit	**Manufacturer**
CD69 Microbead kit II, human	Miltenyi Biotec
IOTest Beta Mark TCRβ Repertoire Kit	Beckman Coulter
miRNAeasy Micro Kit	Qiagen
MinElute PCR Purification Kit	Qiagen
QIAquick PCR purification kit	Qiagen
PCR-clean-up Gel extraction	MACHEREI-NAGEL
TruSeq DNA PCR-Free Library Prep	Clontech
Masenvirus IgG ELISA	IBL International
Masenvirus µ-capture IgM ELISA	IBL International
Mumpsvirus IgG ELISA	IBL International
Mumpsvirus IgM ELISA	IBL International
Rubella-Virus IgG ELISA	IBL International
Rubella-Virus µ-capture IgM ELISA	IBL International
Tetanus IgG ELISA	IBL International

2.1.2 Buffers and media

Buffer/Medium	Preparation
1X BD FACS Lysing solution	10X BD FACS Lysing solution 1:10 in H_2O
1X BD FACS Perm solution 2	10X BD FACS Perm solution 2 1:10 in H_2O
1X Lyse/Fix Solution	5X Lyse/Fix solution 1:5 in H_2o
1X Annexin V Binding Buffer	20X Annexin V Binding Buffer 1:20 in H_2O
Cell culture medium - RPMI Complete	RPMI Medium 1640 - GlutaMAX 100 U/ml penicillin, 100 µg/ml streptomycin 0.5 M HEPES 5% human AB serum
Cell culture medium stromal cell line - RPMI Complete	RPMI Medium 1640 - GlutaMAX 100 U/ml penicillin, 100 µg/ml streptomycin 10% FCS
HEPES 1M	238,3 g HEPES 100 mL H_2O
PBS	0.2g KCl; 0.2g KH2PO4; 8g NaCl; 1.15g Na2HPO4 x 2 H_2O; + H_2O (final V = 1L)
PBS/BSA/EDTA	PBS + 0.2g/L BSA; 2mM EDTA
Expansion medium – X-VIVO Complete	X-VIVO 15 Chemically defined medium 100 U/ml penicillin, 100 µg/ml streptomycin 0.5 M HEPES 5% human AB serum

2.1.3 Antigens

Antigen	Type of antigen	Manufacturer	Concentration
CMV-pp65	Recombinant protein	Miltenyi Biotec	38µg/ml
Measles	Virus Lysate	Microbix Biosystems, Inc.	5µg/ml
Mumps	Virus Lysate	Microbix Biosystems, Inc.	10µg/ml
Rubella	Virus protein	Microbix Biosystems, Inc.	5µg/ml
SEB	Staphylococcus enterotoxin B	Sigma-Aldrich	1µg/ml
Tetanus Toxoid	Toxoid	National Institute for Biological Standards and Control	1 LF/mL

2.1.4 Antibodies

Antigen	Fluorophore	Clone	Manufacturer
Annexin V	FITC		Miltenyi Biotec
Bcl2	PE	100	Biolegend
Bim	Pure/A647 conj.	Y36	Abcam/House conj.
CD3	APC-H7	SK7	BD Biosciences
CD3	PerCP	SK7	Miltenyi Biotec
CD3	Cy5	UCHT-1	House conjugate
CD3	FITC	OKT3	House conjugate
CD4	PECy5.5	SK3	eBioscience
CD4	BV650	OKT4	Biolegend
CD4	PE	TT1	House conjugate
CD8	BV875	RPA-T8	Biolegend
CD8	APC-Cy7	HIT8a	Biolegend
CD8	PE-Cy7	GN11/B4D7	House conjugate
CD8	FITC	GN11/B4D7	House conjugate
CD8	Cy5	GN11/B4D7	House conjugate
CD14	V500	HCD14	BD Bioscience

CD14	Cy5	TM1	House conjugate
CD19	PO	BV12	House conjugate
CD19	VioBlue	LT19	Miltenyi Biotec
CD25	APC	4E3	Miltenyi Biotec
CD27	BV605	LI28	BD Bioscience
CD45	FITC	HI30	Biolegend
CD45RA	PE-Cy7	HI100	Biolegend
CD45RA	BV570	HI100	Biolegend
CD45RA	BV605	HI100	Biolegend
CD45RO	PE-Cy7	UCHL1	Biolegend
CD56	PE	HCD56	Biolegend
CD69	BV421	FN50	Biolegend
CD69	APC-Cy7	FN50	Biolegend
CD69	PE-CF594	FN50	BD Biosciences
CD127	PE	REA614	Miltenyi Biotec
CD137	FITC	484	eBioscience
CD154	BV421	24-31	Biolegend
CD185 (CXCR5)	APC	REA103	Miltenyi Biotec
CCR7	A488	G043H7	Biolegend
CLA	A647	HECA-452	Biolegend
IFNγ	PE-Cy7	4S.B3	Biolegend
IL-2	FITC	MQ1-17H12	Biolegend
IL-2	APC-H7	MQ1-17H12	Biolegend
Ki-67	PE	20Raj1	eBioscience
Mcl1	A488	Y37	abcam
NOXA	Pure/A405 conj.	114C307	Abcam/House conj.
PD1	BV786	EH12:2H7	Biolegend
TNFα	APC	MAb11	BD Pharminogen
TNFα	PE	2-17-9-E11	House conjugated

2.1.5 Consumables

Product	Manufacturer
BD Safety-Lok™ blood collection set 21G	BD Biosciences
BD Vacutainer® 170 U/mL Li-Hep 10 mL	BD Biosciences
Conical 50 mL tubes	Sarstedt
Conical 15 mL tubes	Sarstedt
Culture flasks (25 cm^2, 75 cm^2)	Cellstar
Eppendorf tubes 0.5, 1.5, 2.0 mL Safe-Lock™	Eppendorf
FACS tubes round-bottom PS 5 mL	BD Biosciences
Greiner CELLSTAR 24 well cell culture plates	Sigma-Aldrich
Greiner CELLSTAR 48 well cell culture plates	Sigma-Aldrich
Greiner CELLSTAR 96 well cell culture plates	Sigma-Aldrich
Greiner CELLSTAR 96 well cell culture plates (round bottom)	Sigma-Aldrich
LS magnetic separation columns	Miltenyi Biotec
Pipettes (2µl, 10 µl, 200 µl, 1000 µl)	Eppendorf
Pre-separation filters, 30 µm	Miltenyi Biotec
Serum tubes	BD Biosciences
0,2 mL PCR tubes	Eppendorf

2.1.6 Primers for TCR sequencing

Primer name	Oligo Sequence	Manufacturer
UMI	AAGCAGUGGTAUCAACGCAGAGU NNNN U NNNN U NNNNN UCTT gggg	Metabion
Na-SB2-M1_V2	CGA GCG TGA CGA CGA CAG TAG TCG TGG TAT CAA CGC AGA GT	Metabion
Na-SB4-M1_V2	CGA GCG TGA CGA CGA CAG TCA TCG TGG TAT CAA CGC AGA GT	Metabion
Na-SB5-M1_V2	CGA GCG TGA CGA CGA CAG GAT TCG TGG TAT CAA CGC AGA GT	Metabion
Na-SB6-M1_V2	CGA GCG TGA CGA CGA CAG GTC TTG TGG TAT CAA CGC AGA GT	Metabion
Na-SB7-M1_V2	CGA GCG TGA CGA CGA CAG AGT CTG TGG TAT CAA CGC AGA GT	Metabion
Na-SB8-M1_V2	CGA GCG TGA CGA CGA CAG ACT TCA GTG GTA TCA ACG CAG AGT	Metabion
Na-SB9-M1_V2	CGA GCG TGA CGA CGA CAG ATC CTA GTG GTA TCA ACG CAG AGT	Metabion

Na-SB10-M1_V2	CGA GCG TGA CGA CGA CAG CAA CTT GTG GTA TCA ACG CAG AGT	Metabion
Na-SB11-M1_V2	CGA GCG TGA CGA CGA CAG CCT AAT GTG GTA TCA ACG CAG AGT	Metabion
Na-SB12-M1_V2	CGA GCG TGA CGA CGA CAG CGG TCT GTG GTA TCA ACG CAG AGT	Metabion
Na-SB13-M1_V2	CGA GCG TGA CGA CGA CAG CTC GGT GTG GTA TCA ACG CAG AGT	Metabion
Na-SB14-M1_V2	CGA GCG TGA CGA CGA CAG GCT CTG GTG GTA TCA ACG CAG AGT	Metabion
Na-SB16-M1_V2	CGA GCG TGA CGA CGA CAG TAA TCC GTG GTA TCA ACG CAG AGT	Metabion
Na-SB17-M1_V2	CGA GCG TGA CGA CGA CAG TCT GGC GTG GTA TCA ACG CAG AGT	Metabion
Na-SB18-M1_V2	CGA GCG TGA CGA CGA CAG TGG CTC GTG GTA TCA ACG CAG AGT	Metabion
Na-SB19-M1_V2	CGA GCG TGA CGA CGA CAG TTC AAC GTG GTA TCA ACG CAG AGT	Metabion
Na-SB20-M1_V2	CGA GCG TGA CGA CGA CAG GTC TCG GTG GTA TCA ACG CAG AGT	Metabion
N2Na	NNC GAG CGT GAC GAC GAC AG	Metabion
N3Na	NNN CGA GCG TGA CGA CGA CAG	Metabion
N4Na	NNN NCG AGC GTG ACG ACG ACA G	Metabion
bc1R	CAG TAT CTG GAG TCA TTG A	Metabion
bc2R	TGC TTC TGA TGG CTC AAA CAC	Metabion
bc3R-SB2r	TAG TCA CAC STT KTT CAG GTC CTC	Metabion
bc3R-SB4r	TCA TCA CAC STT KTT CAG GTC CTC	Metabion
bc3R-SB5r	GAT TCA CAC STT KTT CAG GTC CTC	Metabion
bc3R-SB6r	GTC TTA CAC STT KTT CAG GTC CTC	Metabion
bc3R-SB7r	AGT CTA CAC STT KTT CAG GTC CTC	Metabion
bc3R-SB8r	ACT TCA ACA CST TKT TCA GGT CCT C	Metabion
bc3R-SB9r	ATC CTA ACA CST TKT TCA GGT CCT C	Metabion
bc3R-SB10r	CAA CTT ACA CST TKT TCA GGT CCT C	Metabion
bc3R-SB11r	CCT AAT ACA CST TKT TCA GGT CCT C	Metabion
bc3R-SB12	CGG TCT ACA CST TKT TCA GGT CCT C	Metabion
bc3R-SB13	CTC GGT ACA CST TKT TCA GGT CCT C	Metabion
bc3R-SB14	GCT CTG ACA CST TKT TCA GGT CCT C	Metabion
bc3R-SB16	TAA TCC ACA CST TKT TCA GGT CCT C	Metabion
bc3R-SB17	TCT GGC ACA CST TKT TCA GGT CCT C	Metabion
bc3R-SB18	TGG CTC ACA CST TKT TCA GGT CCT C	Metabion
bc3R-SB19	TTC AAC ACA CST TKT TCA GGT CCT C	Metabion
bc3R-SB20	GTC TCG ACA CST TKT TCA GGT CCT C	Metabion

2.1.7 Equipment

Product	Manufacturer
BD Aria II cell sorter	BD Biosciences
BD LSR II flow cytometer	BD Biosciences
Centrifuge 5810R	Eppendorf
SPECTRAmax PLUS 384	Molecular Devices
Heraeus Biofuge Fresco 17	Thermo Scientific
Heraeus Biofuge Fresco 21	Thermo Scientific
Heraeus Multifuge 3SR	Thermo Scientific
HERAsafe biosafety cabinet	Thermo Scientific
Hypoxia biosafety cabinet	BioSpherix
MACSQuant analyzer	Miltenyi Biotec
Miseq 500	Illumina
NanoDrop	Peqlab
Standard CO_2 incubator	Binder
Vacuum pump BVC 21	Vacuubrand
Vortex Mixer	VWR International
Water bath	Memmert

2.1.8 Software

Software	Provider
BD FACSDiva 6	BD Biosciences
MACSQuantify software	Miltenyi Biotec
Flowjo 9.9.4 / 10.2	Tree Star
GraphPad Prism 5.x	GraphPad Software

2.2 Methods

2.2.1 Stromal cells culture

The human stromal cell line HS5 was kindly provided by Dr. Torok-Storb (FHCRC, Seattle, WA, USA). Cells were thawed following the habitual protocol and maintained through all the experimental time in 75 cm^2 culture flasks filled with 10 mL of RPIM + 10% FCS + 1% PenStrep. Cells were passaged when reaching 80% of confluence by tripsinization. After 10-15 passages, cultured stromal cells were discarded and new aliquots thawed in order to assure their support on survival.

The day before the co-culture experiment, stromal cells were tripsinized, and 5 x 10^3 cells were plated in 96 well plates. For transwell experiments, 2 x 10^4 stromal cells were plated in 24-well plates.

2.2.2 Sample collection and cell preparation

Paired BM and PB samples were collected from anonymous systemically healthy adult donors undergoing hip replacement operations. All samples were obtained with local ethical committee (Ethikkommission der Charité-Univerisitätsmedizin Berlin) approvals and informed consent in accordance with the Declaration of Helsinki. Freshly obtained samples were subjected to immediate preparation. PB and BM mononuclear cells were isolated by density gradient sedimentation using Ficoll-Paque Plus. PB was diluted 1:1 with PBS (room temperature). BM samples were thoroughly washed with room temperature PBS and filtered through using a 70µm filter. Afterwards each cell suspension was carefully layered on Ficoll in proportion 35ml cell suspension to 15ml Ficoll and centrifuged at 2000rpm for 20 minutes at room temperature (acceleration = 7; deceleration = 0). The upper plasma layer was removed and the intermediate lymphocyte layer transferred into a clean 50ml falcon tube. PBMCs/BMMCs were washed with cold PBE and centrifuged for 10 minutes at 300xg and 4°C. The supernatant was discarded and the cell pellet resuspended in 50ml cold PBS/BSA/EDTA. To dispose of residual granulocytes the cells were ultimately centrifuged for 15 minutes at 180xg and 4°C, the supernatant was discarded.

2.2.3 FACS sorting of memory T cells subsets

Isolated cells were surface stained with the following antibody mix [anti-CD4 (OKT4), anti-CD8 (HIT8a), anti-CD45RA (HI100), anti-CD45RO (UCHL1), anti-CD69 (FN50), anti-CD25 (4A3), anti-CD19 (BV12), anti-CD14 (HCD14)] for 10 minutes at 4°C. After that, cells were washed with PBE and centrifuged 5 minutes at 1200 rpm. Supernatant was discarded and cells were re-suspended in PBE according with the cell numbers for sorting. Memory T cell subsets were sorted in a FACS Aria II using the following gating strategy:

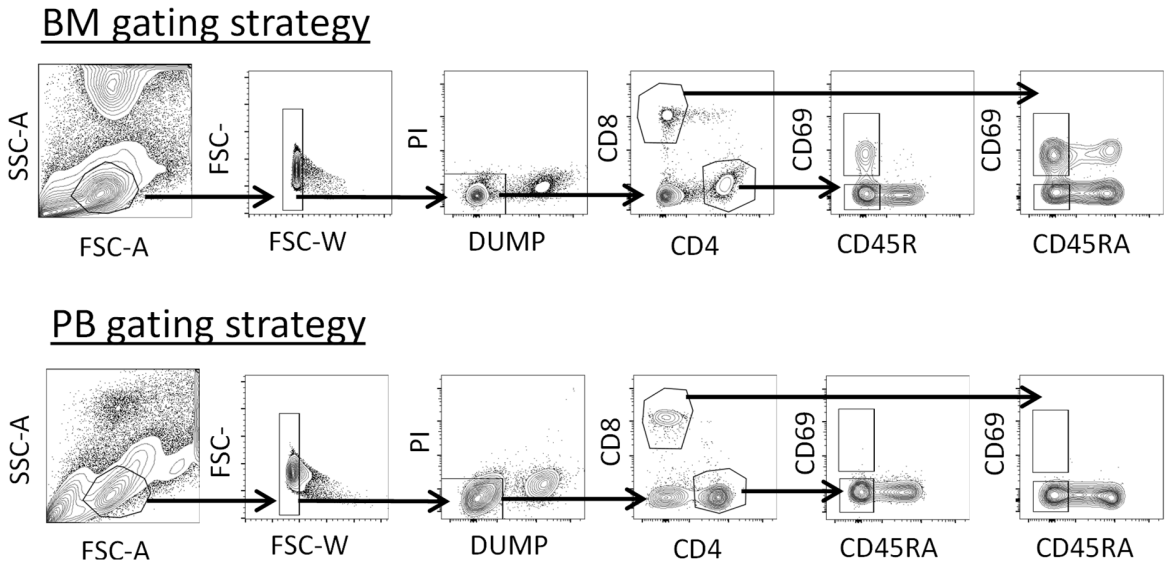


Figure 2-1: Gating strategy for the sorting of PB and BM memory T cell populations. Memory T cell subsets were sorted in a FACS Aria II using the following gating strategy: (1) $PI^-CD14^-CD19^-CD4^+CD25^-CD45RA^-CD45RO^+CD69^+$, (2) $PI^-CD14^-CD19^-CD4^+CD25^-CD45RA^-CD45RO^+CD69^-$, (3) $PI^-CD14^-CD19^-CD8^+CD25^-CD45RA^-CD45RO^+CD69^+$, (4) $PI^-CD14^-CD19^-CD8^+CD25^-CD45RA^-CD45RO^+CD69^-$.

2.2.4 Cell culture conditions

After FACS sorting, cells were centrifuged 5 minutes at 1200 rpm and re-suspended in complete RPMI, in a final concentration of $5x10^4$cell/mL. 100 uL of cells were plated in 96 well plates previously prepared with 100 uL of the tested conditions in each experiment (IL7, IL15, IL6, IL33, TNFα, TGFβ and HS5). Cells were cultured in 96 well plates at 37°C, 5%CO_2 and 21% (NOX) or 4% (HOX) O_2, depending on the experiment. For transwell experiments, the same amount of cells were plated into an insert and placed in 24 well plates previously seeded with stromal cells.

2.2.5 Cell survival assessment / intracellular staining of pro/anti-apoptotic proteins

Cell survival and/or expression of pro/anti-apoptotic markers were measured at days 0, 1, 3 and 5 of culture. Cell survival was measured by ANNV/PI staining following manufacturer´s instructions. Briefly, cells were harvested from wells and transferred to 96-well plated with U bottom, after that, plates were centrifuged 5 min at 1200 rpm and cells were resuspended in the following mix, prepared in ANNV binding Buffer [ANNV, anti-CD127 (REA614), anti-CD3 (UCHT-1)] and incubated for 15 min at room temperature. After that, plates were centrifuged 5 min at 1200 rpm and resuspended in 150 uL of ANNV Binding Buffer. PI was added just before measuring to all samples and they were analyzed by MACS Quant.

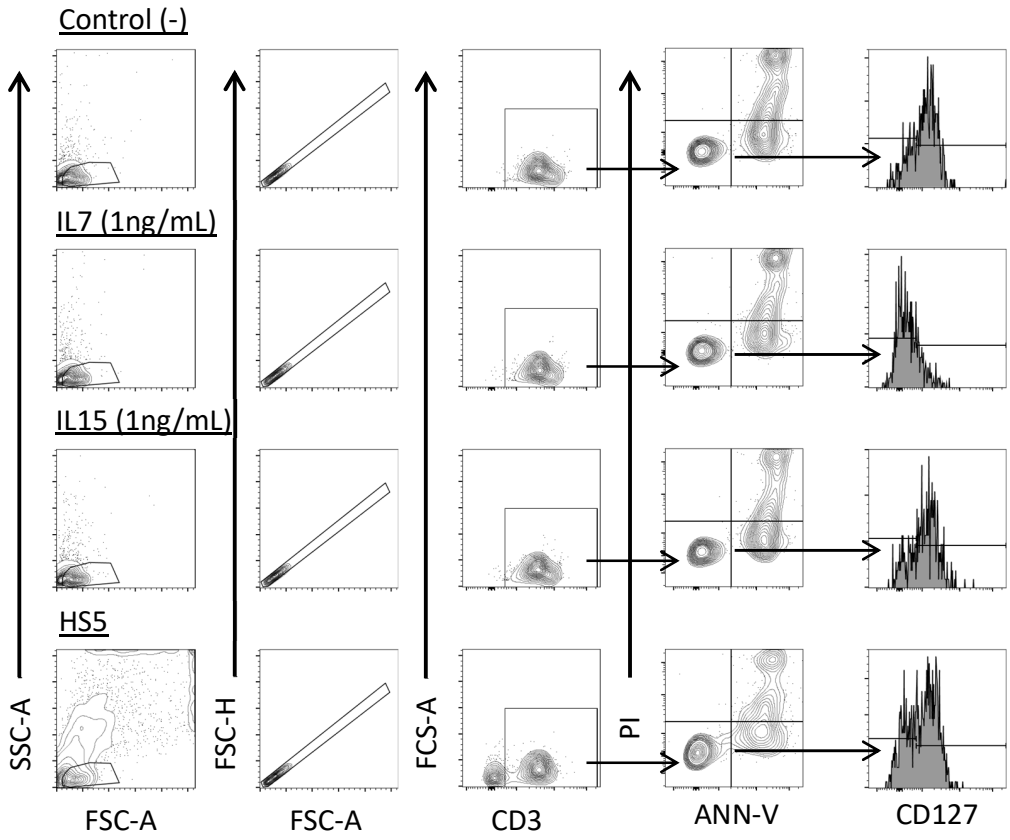


Figure 2-2: Gating strategy followed during survival experiments and representative dot plot of all conditions analyzed. First, lymphocytes were gated regarding their FCS-SSC properties and duplets were excluded. Afterwards, I gated in $CD3^+$ T cells and analyzed the percentages and numbers of surviving cells by gating on Annexin V^- PI^- cells. The expression of CD127 was examined in living cells, determining the mean fluorescence intensity (MFI) of the receptor in each cell subset during the different culture conditions and times.

For the intracellular staining of pro/anti-apoptotic proteins, cells were fixed with 2% PF for 10 minutes at room temperature. Fixed cells were washed with PBE and centrifuged 5 minutes at 500 xg. After that, cells were incubated with Permeabilization Buffer A 30 minutes on ice and stained with the following antibody mix [anti-CD3 (SK7), anti-Bcl2 (100), anti-Bim (Y36), anti-Mcl1 (Y37), anti-NOXA (114C307)] for 30 minutes at room temperature. After that, cells were washed with PBE, centrifuged 5 minutes at 500xg and resuspended in 70 uL PBE. Samples were analysed by MACS Quant.

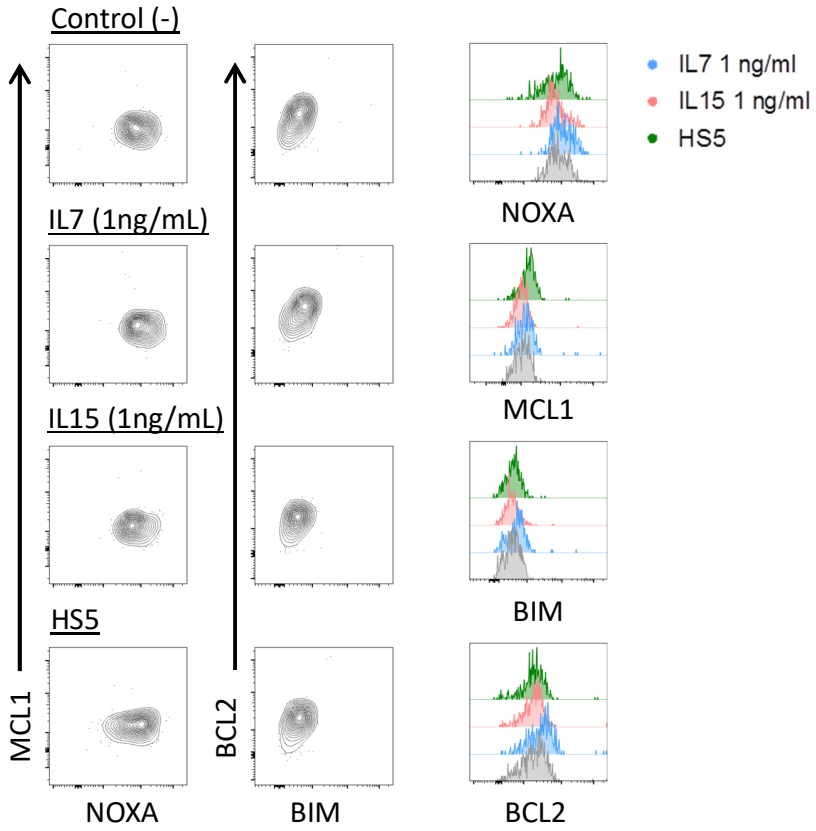


Figure 2-3: Intracellular analysis of pro-and anti-apoptotic markers at day 3 of culture in all conditions analyzed. First, lymphocytes were gated regarding their FCS-SSC properties and duplets were excluded. Afterwards, I gated in $CD3^+$ T cells and analyzed the expression of BCL-2, BIM, MCL-1 and NOXA in all cell subsets regarding the different cell culture conditions tested.

2.2.6 Isolation of CD69$^{+/-}$ cells and CFSE/efluo670 labelling

Mononuclear cells from PB and BM paired samples were isolated as explained in 2.2.1.2. After isolation, BM cells were sorted for CD69 expression using CD69 Microbeads Kit II (Miltenyi), following manufacturer´s instructions. Briefly, mononuclear cells were resuspended in PBS/BSA/EDTA with the final concentration of 10^7 cells per 40µl buffer, 2µl of FcR Blocking Reagent and 10µl of CD69-Biotin were added and the cell suspension was incubated for 15 minutes at 4°C. Afterwards, 30 µL of PBS/BSA/EDTA and 20 µL of anti-Biotin Microbeads were added to the cell suspension and samples were incubated for an additional 15 minutes at 4°C. Finally, cells were washed with 20-fold volume of PBS/BSA/EDTA and centrifuged for 10 minutes at 300xg and 4°C. The supernatant was discarded and the cell pellet was resuspended in 500µl PBS/BSA/EDTA (if cell number exceeded 10^8 cells, 2ml of buffer and two MACS LS separation columns were used). LS separation columns and 30µm filter were rinsed with 3ml PBS/BSA/EDTA and the cells were loaded onto the column. After the cell suspension has passed the column, the column was washed with 3x3ml PBE and magnetically labeled cell fraction was flushed out of the column with 5ml buffer. The flow-through and positive fraction were collected and purity was assessed by CD69 staining and measurement at MACSQuant.

After Magnetic Activated Cell Surface Sorting (MACS sorting) of the CD69$^+$ and CD69$^-$ fractions, mononuclear cells were washed with PBS and spun down at 300xg and 4°C for 10 minutes. Subsequently, the cells were resuspended in PBS at final concentration 10^7 cells/ml. Stock solutions of CFSE and efluo670 were diluted in PBS to the double working concentration and drop by drop added to the cell suspension while vortexing (final concentration 2.5µM). Afterwards cells were incubated for 15 minutes at 37°C and the staining was quenched by adding serum-containing RPMI medium and incubation for further 10 minutes at 37°C. Ultimately, cells were spun down and washed twice with RPMI plus 5% human AB-Serum. Staining of different fractions was assessed in MACSQuant, and both fractions were mixed for antigen specific T cell stimulation.

2.2.7 Antigen specific T cell stimulation

To access antigen-specific memory T cells 10^7 PB and/or BM mononuclear cells were stimulated in 1ml complete RPMI medium with antigens described in section 2.1.3. Cells were stimulated for 12 hours with protein- or peptide-antigens. To block cytokine secretion,

brefeldin A (5 µg/mL) was added after first 2 hours of stimulation. As a high control a fraction of cells was stimulated the superantigen SEB (1 µg/mL) for 12 hours. Subsequently, stimulated cells were harvested, stained for surface antigens followed by fixation, permeabilisation and intracellular cytokine staining.

2.2.8 Cell surface and intracellular cytokine staining

Phenotype of PB and BM resident T cells was analyzed using cell surface marker staining. Mononuclear cells were washed and resuspended in 100µl of cold PBS/BSA/EDTA with the cell density of up to $5x10^6$ cells in 100µl buffer. Subsequently, staining antibodies in combination with Fc Blocking reagent to prevent unspecific Fc Receptor binding were added. Mononuclear cells were stained for 10 minutes in the dark at 4°C, and in case of chemokine receptor staining for 15 minutes in the dark at 37°C. After this the cells were washed with cold PBS/BSA/EDTA and acquired using LSR Fortessa. Data analysis was performed using FlowJo (Treestar) software.

To access the cytokine production by in vitro activated T lymphocytes, intracellular cytokine staining was performed. Pre-stimulated cells were harvested, washed with cold PBS/BSA/EDTA and spun down at 300xg and 4°C for 10 minutes. The supernatant was discarded and the cells were fixed for 10 minutes with 100µl 1X Lysing solution (BD Biosciences) at room temperature. Subsequently fixed cells were washed with 2ml cold PBS and centrifuged down at 490xg and 4°C for 10 minutes. To permeabilize the cells FACS Permeabilizing Solution 2 (BD Biosciences) was used. 100µl of 1x Permeabilizing Solution 2 Buffer were added to fixed cells and incubated for 10 minutes at room temperature in the dark. After washing the cells with PBS (490xg, 4°C, 10 minutes) antibody staining was performed. Final staining volume was adjusted to 100µl per max. $5x10^6$cells. Cells were stained for 30 minutes at room temperature in the dark, washed with cold PBS (490xg, 4°C, and 10 minutes) and analysed using LSR Fortessa and FlowJo (Treestar). At least $1×10^6$ lymphocytes were acquired.

2.2.9 Quantitative TCRVβ chain repertoire analysis

Quantitative analysis of TCRVβ chain repertoire was performed using IOTest Beta Mark TCRVβ Repertoire Kit according to manufacturer's instructions. Additionally, to differentiate distinct T cell subsets anti-CD3 (SK7), anti-CD4 (OKT4), anti-CD8 (HIT8a),

anti-CD45RA (HI100) and anti-CD69 (FN50) surface stainings were performed. Samples were acquired using LSRFortessa and analyzed using FlowJo (Treestar). Following TCRβ chain variants composing up to 70% of TCRβ chain repertoire were covered[181]: Vβ1, Vβ2, Vβ3, Vβ4, Vβ5.1, Vβ5.2, Vβ5.3, Vβ7.1, Vβ7.2, Vβ8, Vβ9, Vβ11, Vβ12, Vβ13.1, Vβ13.2, Vβ13.6, Vβ14, Vβ16, Vβ17, Vβ18, Vβ20, Vβ21.3, Vβ22, Vβ23.

2.2.10 Cell isolation and FACS sorting for TCRβ NGS

Paired PB and BM samples were directly sorted in the different memory T cell subsets or stimulated with different antigens for the sorting of antigen specific memory T cells.

For the sorting of directly ex vivo cell subsets, isolated cells were surface stained with the following antibody mix [anti-CD4 (OKT4), anti-CD8 (HIT8a), anti-CD45RA (HI100), anti-CD45RO (UCHL1), anti-CD69 (FN50), anti-CD25 (4A3), anti-CD19 (BV12), anti-CD14 (HCD14)] for 10 minutes at 4°C. After that, cells were washed with PBE and centrifuged 5 minutes at 1200 rpm. Supernatant was discarded and cells were re-suspended in PBE according with the cell numbers for sorting. 25.000 memory T cell subsets were sorted in a FACS Aria II using the following gating strategy:

(1) $PI^-CD14^-CD19^-CD4^+CD25^-CD45RA^-CD45RO^+CD69^+$,

(2) $PI^-CD14^-CD19^-CD4^+CD25^-CD45RA^-CD45RO^+CD69^-$,

(3) $PI^-CD14^-CD19^-CD8^+CD25^-CD45RA^-CD45RO^+CD69^+$,

(4) $PI^-CD14^-CD19^-CD8^+CD25^-CD45RA^-CD45RO^+CD69^-$.

When sorting antigen specific memory $CD4^+$ T cells, at least 5 x 10^7 mononuclear cells (both from PB and BM) were stimulated for 12 h with tetanus toxoid (TT) in the presence of 1 µg/ml CD40 functional grade pure Ab (Miltenyi Biotec). After stimulation, cells were stained 15 min at room temperature with the following mixture of antibodies [anti-CD3 (UCHT1), anti-CD4 (TT1), anti-CD8 (GN11/134D7), anti-CD19 (BU12), anti-CD14 (TM1), anti-CD45RA (HI100), anti-CD69 (FN50) and anti-CD154 (24-31)]. 2500 antigen-specific T cells were sorted by using an Aria II FACS sorter according the following gate strategy

- $PI^-CD19^-CD14^-CD3^+CD8^-CD4^+CD45RA^-CD154^+CD69^+$.

Sorted cells were resuspended in 700 µL of Qiazol Reagent and kept at -80°C until RNA extraction.

2.2.11 Preparation of TCRβ-seq library and sequencing

RNA extraction. For total RNA extraction, I used Qiazol reagent in combination with miRNAeasy Micro Kit (Qiagen), following manufacturer´s protocol. RNA concentration and quality were determined by Bioanalyzer picogram analysis.

cDNA synthesis. RNA solution was combined with 2μM 5'-template switch adapter SmartNNNa and 2μM β-chain reverse primer (section 2.1.6). After that, a premix containing SMARTScribe buffer, 20 mM DTT, 10 mM each dNTP and 20 U/μL and SMARTScribe reverse transcriptase (100U/μL). The solutions were incubated for 90 min at 42°C for cDNA priming, synthesis and template switch. The reaction was stopped by incubating further 15 min at 70°C and the deoxyuridine-containing template switch adapter was removed by treating the cDNA with uracil-DNA glycosylase (New England BioLabs, 5U/μL) for 40 min at 37°C. cDNA products were purified using MinElute PCR purification Kit (Qiagen), following manufacturer´s instructions.

PCR amplification. For the first PCR amplification, all obtained cDNA was processed. I prepared a mix containing 5X buffer for Q5 DNA polymerase (New England Biolabs), 0.2 mM of each dNTP, 0.5 μM forward barcoded primers annealing the SmartNNNa adapter and 0.5 μM reverse primer for β-chain amplification (section 2.1.6) , Q5 DNA polymerase (New England Biolabs) and sterile water to a final volume of 50 μL. PCR reaction was performed using the following optimized cycling conditions: 94°C for 2 min, then 94°C for 10s, 55°C for 10s and 72°C for 50s (21 cycles), with a final elongation at 72°C for 2 min. PCR products were purified using QIAquick PCR purification kit (Qiagen).

Each 1st PCR reaction was divided into several reactions for the second step PCR. Reaction mix included 5X buffer for Q5 DNA polymerase (New England Biolabs), 0.2 mM of each dNTP, 0.5 μM forward primers annealing the adapter and 0.5 μM barcoded reverse primers for β-chain constant gene segment (section 2.1.6), Q5 DNA polymerase (New England Biolabs) and sterile water to a final volume of 50 μL. PCR reaction was performed using the following optimized cycling conditions: 94°C for 2 min, then 94°C for 10s, 55°C for 10s and 72°C for 50s (18 cycles), with a final elongation at 72°C for 2 min. PCR products were purified using QIAquick PCR purification kit (Qiagen).

Final products were purified using PCR-clean-up Gel extraction (MACHEREI-NAGEL) and stored at -20°C until adaptor ligation and sequencing.

PCR product concentration in each library was determined using a Qubit fluorometer (Invitrogen). PCR products were mixed together in an equal ratio, and Illumina adapters were ligated according to the manufacturer´s protocol. After adaptor ligation, pooled libraries were further purified using PCR-clean-up Gel extraction (MACHEREI-NAGEL).

2.2.12 Illumina sequencing and data processing

2x150bp paired-end sequencing of TCRβ amplicons was performed on the Illumina NextSeq 500 platform. Inline barcodes were demultiplexed and Illumnia Adapters removed by Illuminas bcl2fastq 1.8.4 software. De-multiplexing of sample barcodes as well as the extraction of the CDR3-sequences were performed by migec 1.2.4 using the unique molecular identifier guided assembling and error correction with default setting, minimal number of 5 reads per UMI and blast. Downstream analyses were performed based on functional CDR3 nucleotide sequences, i.e. not containing a stop codon or frame shift, using own scripts. Technical replicates were merged if specified. Diversity of the TCRβ repertoires was compared using the Efron and Thisted index and hill numbers. Similarity of TCRß repertories for CD4, CD8, CD69^{+} and CD69^{-} samples from BM and peripheral blood were evaluated using circle plots with merged technical replicates and clusterisation by cosine distance and WARD as linkage criteria.

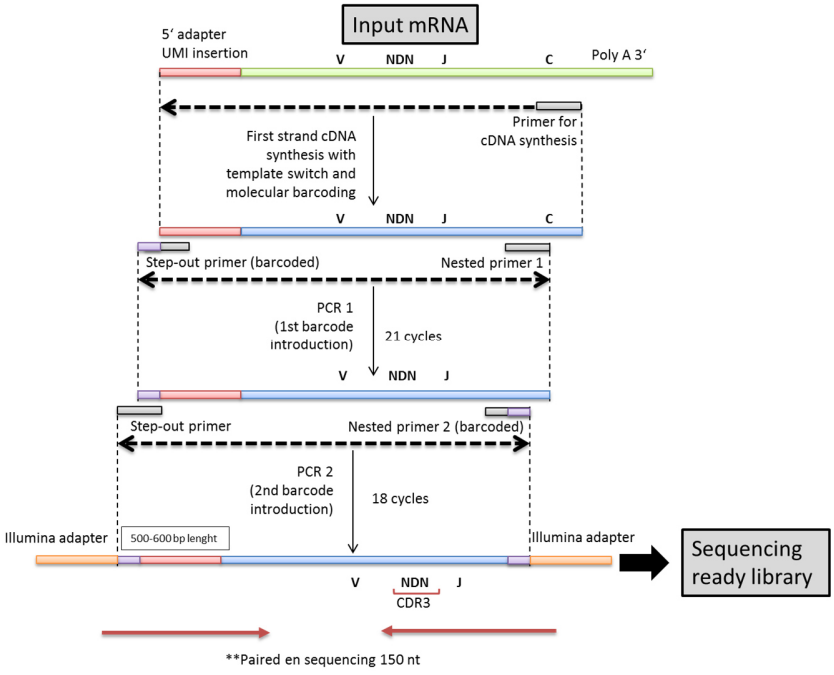


Figure 2-4: Schematic strategy for preparation of TCRβ libraries. Process is explained in section 2.2.11.

2.2.13 MMR Vaccination and study cohort

Approval for this study was obtained from the ethics committee of the Faculty of Medicine-Charité, Berlin, Germany (EA1/303/12 and EA1/342/14). After informed consent and prior vaccination, 24 volunteers were screened for the presence of neutralizing antibodies against measles-mumps and rubella in their serum (Labor Berlin) but non-detectable or low numbers of antigen-specific T cells in blood circulation by flow cytometry analysis. Finally, 11 healthy adults (age ± SEM= 34.8±6.2; 4male/7 female; table S6.3) were vaccinated with a measles-mumps-rubella (MMR) live attenuated vaccine (Priorix®, GSK, Germany). 50 mL of blood were drawn immediately before vaccination (day 0), and at 6 further time points (16 hours and days 1, 2, 3,7 and 14) or 2 further time points (days 1 and 14) after vaccination in EDTA tubes (BD Biosciences, Plymouth, U.K.). Collected blood was processed and analysed directly. Serum from all donors was collected at days 0, 1, 3 and 14 after vaccination in serum gel tubes (BD Biosciences, Plymouth, U.K.) and stored at -20°C until use.

2.2.14 Measles, mumps, rubella and tetanus toxoid (TT) serology

Specific IgM and IgG responses against measles, mumps and rubella, and IgG responses against TT were analyzed in days 0, 1, 3 and 14 of all vaccinees. Serum was analyzed with the measles, mumps, rubella and TT IgM and IgG-ELISAS (IBL International Gmbh, Hamburg, Germany) following manufacturer´s instructions.

2.2.15 Isolation of PB mononuclear cellsBMC)

Peripheral blood mononuclear cells were isolated by density gradient sedimentation using Ficoll-Paque Plus. PB was diluted 1:1 with PBS (room temperature). Each cell suspension was carefully layered on Ficoll in proportion 35ml cell suspension to 15ml Ficoll and centrifuged at 2000rpm for 20 minutes at room temperature (acceleration=7; deceleration=0). The upper plasma layer was removed and the intermediate lymphocyte layer transferred into a clean 50ml falcon tube. PBMCs/BMMCs were washed with cold PBS/BSA/EDTA and centrifuged for 10 minutes at 300xg and 4°C. The supernatant was discarded and the cell pellet resuspended in 50ml cold PBS/BSA/EDTA. To dispose of residual granulocytes the cells were ultimately centrifuged for 15 minutes at 180xg and 4°C, the supernatant was discarded.

2.2.16 Analysis of absolute numbers of leukocyte populations

To determine absolute counts of the analysed leukocyte populations, 50 µL of freshly drawn whole blood with 50 µL of antibody mixture [anti-CD45 (HI30), anti-CD56 (HCD56) and anti-CD3 (SK7), anti-CD19 (LT19), anti-CD4 (SK3) anti-CD8 (GN11/134D7) and anti-CD14 (TM1)]. The staining was performed in the presence of FcR-blocking reagent (Miltenyi Biotec). After staining, erythrocytes were lysed by adding 500 µL of Buffer EL (Qiagen) and incubating 30 min at 4°C. Samples were directly analysed on a MACSQuant (Miltenyi Biotec), and counts of leukocyte populations measured were used for calculations of total cell populations determined in further FACS panels.

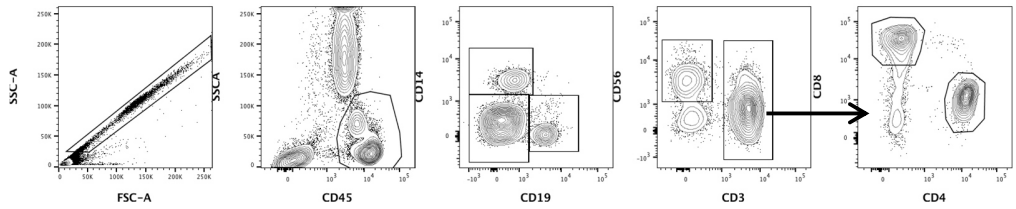


Figure 2-5: Gating strategy for the whole blood cell counting panel measured in MACSQuant. After exclusion of aggregates, cells from lysed whole blood were gated on $CD45^+$ cells. Monocytes ($CD14^+$) and B cells ($CD19^+$) were identified. Cells being neither a monocyte nor a B cell were further distinguished into T cells ($CD3^+$) and NK cells ($CD56^+$): T cells were sub-divided into $CD4^+$ and $CD8^+$ T cells. Calculations of whole blood cell count on other panels were based on measured events of populations determined in this panel.

2.2.17 Identification of MMR-reactive memory $CD4^+$ T cells.

For the identification of antigen-reactive $CD4^+$ T cells, isolated PBMCs were cultured in RPMI 1640 supplemented with 1% GlutaMAX, 100 U/mL penicillin, 100 µg/mL streptomycin (Invitrogen Life Technologies), and 5% (vol/vol) human AB serum (Sigma-Aldrich). Cells were stimulated with measles (5µg/mL) and mumps (5µg/mL) grade 2 antigens, rubella K2S (10 µg/mL) antigen and tetanus toxoid (TT) (1,000 Lethal factor/mL) in the presence of CD28 functional grade (1 µg/mL). The specific reaction was controlled by including cells stimulated only with CD28 and CD28 plus SEB (1 µg/mL). For each condition, at least 5-10 x 10^6 cells were stimulated for 7 h at 37° and 5% CO_2, in the presence of brefeldin A (1 µg/mL) for the last 2 h. After stimulation, cells were transferred to flow cytometry tubes and surface staining was performed for 10 min at 4°C with the

following Ab mixture [anti-CD3 (BW264/56), anti-CD4 (SK3), anti-CD8 (RPA-T8), anti-CD45RA (HI100), anti-CD19 (BU12), anti-CD14 (TM1) and LD-PO]. Cells were fixed by incubating with 100 µL of 1X BD FACS Lysing solution and permeabilized by additional 10 min incubation with 100 µL of 1X BD Perm2 solution. PBMCs were then washed with PBS/BSA and stained for 30 min at room temperature with the following antibody mix [anti-CD154 (24-31), anti-IFNγ (45B3), anti-IL2 (MQ1-17H12), anti-TNFα (Mab11) and anti-KI67 (20Raj1)]. Stained cells were measured using an LSR II flow cytometer (BD Biosciences). The antigen-reactive memory $CD4^+$ T cells were identified as Live/$Dead^-CD19^-CD14^-CD3^+$ $CD4^+CD45RA^-CD154^+$cytokine$^+$ and the antigen-reactive proliferating cells were measured as Live/$Dead^-CD19^-CD14^-CD3^+CD4^+CD45RA^-CD154^+$ Ki-67^+. At least 1 x 10^6 lymphocytes were acquired. An exemplary gating is shown in figure 2.6:

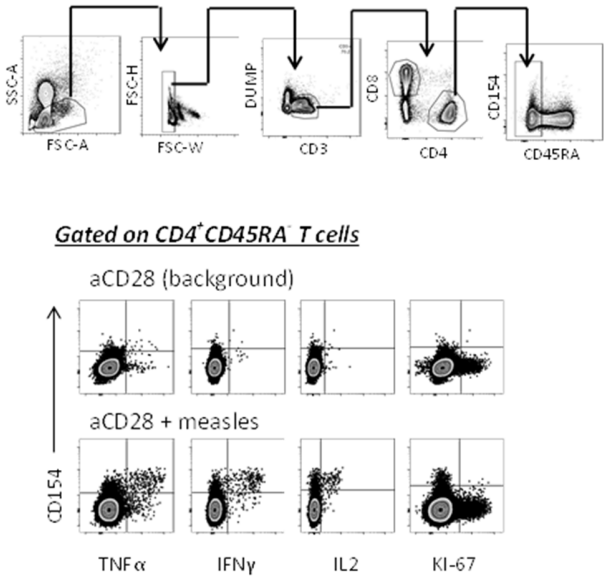


Figure 2-6: Gating strategy for the antigen-reactive memory T cells panel. Activated and cytokine expressing $CD4^+$ T cells were assessed after antigen-specific stimulation of PBMCs at different time points. First, we gated on lymphocytes based on FSC-SSC properties and excluded aggregates. Then we gated on $DUMP^-$ (including dead, $CD19^+$ and $CD14^+$ cells) $CD3^+$ T cells. After distinguishing from $CD8^+$ and $CD4^+$, we analyzed activation marker (CD154); cytokine (IFNγ; IL-2 and TNFα) and proliferation marker (KI-67) expression in $CD4^+$ $CD45RA^-$ T cells. Co-expression of CD154 and cytokines or KI-67 is reported in the results. For assessment of the qualitative $CD4^+$ T cell response, CD154+ CD4+ T cells were further used in Boolean gating approach generating results for all possible cytokine combinations. For each subpopulation, the background (as detected in the anti-CD28 stimulated control samples) was subtracted.

2.2.18 Multiparametric Flow cytometric analysis

Eight- to twelve- colour flow cytometry analysis was performed in directly “ex vivo” isolated cells and antigen-stimulated samples for the analysis of phenotype and activation status. The following anti-human antibodies were used for this purpose [anti-CD3 (BW264/56), anti-CD4 (SK3), anti-CD8 (RPA-T8), anti-CD45RA (HI100), anti-CD69 (FN50), anti-CD127 (REA614), anti-CXCR5 (REA215), anti-Ki-67 (20Raj1), anti-CD137 (484), anti-PD1 (EH12:2H7), anti-CCR7 (G043H7), anti-CD154 (24-31), anti-CD19 (BU12), anti-CD14 (TM1) and fixable Live/Dead PO. Stained cells were acquired in an LSR II flow cytometer (BD Biosciences). An example of the gating strategy used to define the expression of activation and phenotypic markers is depicted in figure 2.7:

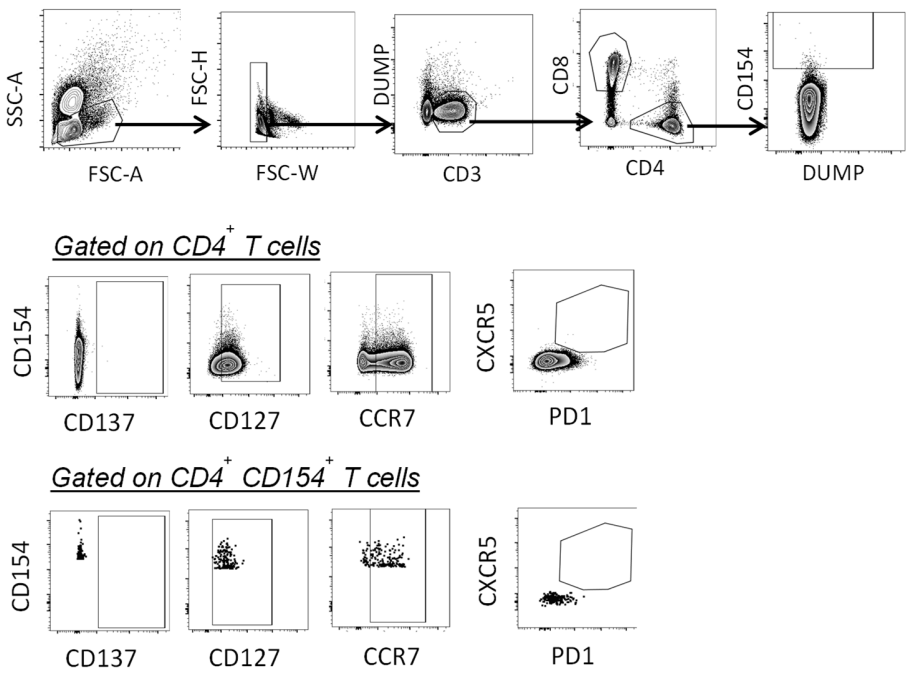


Figure 2-7: Gating strategy for cellular profiling of antigen-reactive CD4+ T cells. Antigen-reactive $CD4^+$ T cells were further phenotypically characterized after antigen-specific stimulation of PBMCs at different time points. First, I gated on lymphocytes based on FSC-SSC properties and excluded aggregates. Then I gated on $DUMP^-$ (including dead, $CD19^+$ and $CD14^+$ cells) $CD3^+$ T cells. After distinguishing from $CD8^+$ and $CD4^+$, I gated on the activation marker (CD154) and assessed the expression of various cell subset defining (CD127; CD137, CCR7, PD-1 and CXCR5) markers.

2.2.19 FACS enrichment of antigen-reactive CD4$^+$ T cells

For enrichment of antigen-specific memory CD4$^+$ T cells, at least 5 x 10^7 PBMCs were stimulated for 7 h with measles grade 2 antigens (5µg/mL) in the presence of 1 µg/ml CD40 functional grade pure Ab (Miltenyi Biotec). After stimulation, cells were stained 15 min at room temperature with the following mixture of antibodies [anti-CD3 (UCHT1), anti-CD4 (TT1), anti-CD8 (GN11/134D7), anti-CD19 (BU12), anti-CD14 (TM1), anti-CD45RA (HI100), anti-CD69 (FN50) and anti-CD154 (24-31)]. Stained cells were purified by using an Aria II FACS sorter according the following gate strategy:

- PI$^-$CD19$^-$CD14$^-$CD3$^+$ CD4$^+$CD45RA$^-$CD154$^+$CD69$^+$.

Measles-specific memory CD4$^+$ T cells sorted at days 0, 1 and 14 after vaccination were further processed for TCR sequencing, and measles-specific memory CD4$^+$ T cells sorted at day 1 after vaccination were used for the generation of antigen-specific T cell lines.

2.2.20 Expansion and re-stimulation of Ag-specific T cell lines

Stimulation of PBMCs and isolation of antigen-reactive memory CD4$^+$ T cells were performed as described above. Isolated CD69$^+$CD154$^+$ CD4$^+$ T cells were cultured with CD3 depleted and irradiated (40 Gy for 35 min) autologous feeder cells in a ratio of 1:100 in 48-well plates in X-VIVO15 (Lonza), supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (Invitrogen Life Technologies), 5% (vol/vol) human AB serum and 200 IU/mL IL-2 (Proleukin®). Cells were expanded for 14 days, changing culture media with containing new IL-2 every 3 days during the first 10 days.

For re-stimulation, freshly isolated autologous PBMCs were labelled with CFSE (1 µM) following manufacturer´s protocol. 2,5 x 10^5 expanded T cells were combined with labeled PBMCs in a ratio of 1:2 and stimulated with different antigens (measles, mumps, rubella, TT and CMV) in the presence of CD28 functional grade pure (1 µg/mL; Miltenyi Biotec) for 6 h, adding brefeldin A (1 µg/mL; Sigma-Aldrich) after the first 2 hours. Cells were stained with surface and intracellular markers as described above for the analysis of CD154 and cytokine expression, using the following antibodies [anti-CD3 (BW264/56; Miltenyi Biotec), anti-CD4 (SK3; eBioscience), anti-CD8 (RPA-T8; Biolegend), anti-CD45RA (HI100; Biolegend), anti-CD19 (BU12, DRFZ), anti-CD14 (TM1, DRFZ), anti-CD154 (24-31), anti-IFNγ (45B3), anti-IL2 (MQ1-17H12; all from Biolegend), anti-TNFα (Mab11; BDPharminogen) and anti-KI67 (20Raj1; eBioscience)].

2.2.21 TCRVβ libraries preparation and data analysis

2500 Measles-specific memory $CD4^+$ T cells were sorted at days 0, 1 and 14 as described above for CDR3 TCRβ library preparation and sequencing. Total RNA extraction and library preparation were performed as explained in section 2.2.11.

2x150bp paired-end sequencing of TCRβ amplicons was performed on the Illumina NextSeq 500 platform. Inline barcodes were demultiplexed and Illumnia Adapters removed by Illuminas bcl2fastq 1.8.4 software. De-multiplexing of sample barcodes as well as the extraction of the CDR3-sequences were performed by migec 1.2.4 using the unique molecular identifier guided assembling and error correction with default setting, minimal number of 5 reads per UMI and blast. Downstream analyses were performed based on functional CDR3 nucleotide sequences, i.e. not containing a stop codon or frame shift, using own scripts. Technical replicates were merged if specified. Diversity of the TCRβ repertoires was compared using the Efron and Thisted index and hill numbers. The TCRβ repertoire for measles-specific clonotypes were evaluated by comparing clonotypes solely appearing on a particular day post vaccination, that is constantly present in both replicates at that day and not appearing in any sample from different day.

2.2.22 Data acquisition, analysis and statistics

FACS data was acquired on LSR II by FACSDiva 6 and on MACSQuant by MACSQuantify Software. All FACS data were properly compensated using matching single-stain and FMO controls[182]. Data was exported into fcs-data files and analyzed with Flowjo 9.7.4 / Flowjo 10 (MacOS Version). Finalized, gated data was exported into data tables. For statistical analysis of any observed data GraphPad Prism 7 was used. To analyze two groups of paired samples, two-tailed Wilkoxon signed-rank test was used. For analyzing more than two groups, ANOVA analysis was applied. For the statistical analysis of The MMR vaccination study, 2-WAY repeated measurement ANOVA with time as a factor was used. P-value of<0.05 was considered significant.

3 Results

3.1 Survival mechanisms of PB circulating versus BM resident memory T cells

3.1.1 Differential survival patterns of PB and BM memory T cell subsets

PB circulating and BM resident memory T cells are subjected to different environmental conditions, and therefore, might present different lifestyles. BM memory T cells are thought to be maintained in specialized stromal niches, which provide them with constant survival signals in order to maintain them alive and in a resting state. On the other hand, memory T cells from blood are in constant circulation, mostly receiving these survival signals during blood circulation or when going through secondary lymphoid organs or tissues. Oxygen levels present in blood differ from the BM microenvironment (from 13% in blood to 6.4% in BM [159]), being a possible factor which could influence memory T cell survival. In order to determine the intrinsic survival capacity as such of memory T cells isolated from PB and BM, I analyzed the survival of $CD4^+$ and $CD8^+$ memory T cell populations when cultured *in vitro.* In addition, we assessed the influence of oxygen levels in memory T cell survival by culturing the cells in RPMI supplemented with %5 AB serum, in the presence of 21% or 4% O_2 (Figure 3.1).

When culturing cells under 21% O_2, both $CD4^+$ and $CD8^+$ $CD69^+$ BM memory T cells showed reduced survival at days 3 and 5 of culture compared to BM $CD69^-$ and PB $CD69^-$ memory T cells. On average, 60% and 52% of $CD69^-$ memory $CD4^+$ T cells of PB and BM were still alive at day 3 of culture, whereas only 45% of $CD69^+$ BM memory $CD4^+$ T cells survived in medium alone. Similarly, the average survival of memory $CD8^+$ T at day 3 were 47%, 48% and 36% for $CD69^-$ PB, $CD69^-$ BM, and $CD69^+$ BM memory T cells, respectively. When cultured in 4% O_2, survival of memory T cells was different. On average, at day three of culture, only 45% of PB memory $CD4^+$ T cells were alive, whereas 52% of $CD69^-$ BM and 56% of $CD69^+$ BM memory T cells survived. All cell subsets of $CD8^+$ memory T cells analyzed showed a similar survival at day 3 of culture at ~55%.

In order to define the half-lives in hours for memory T cells, I determined time where 50% of the population was still alive compared to the starting cell numbers at day 0 of culture. In concordance with the results obtained from the survival curves, under 21% O_2, PB

memory CD4⁺ T cells showed a significantly increased half-life compared to the BM counterparts (p=0.0033 and p=0.0458 compared to BM CD69⁺ and BM CD69⁻ respectively). CD69⁺ BM memory T cells displayed the shortest half-life (Figure 3.1). Similar tendency was observed in the memory CD8⁺ memory T cell populations, where CD69⁻ and CD69⁺ BM memory CD8⁺ T cells showed a reduced half-life compared to the PB counterparts. In particular, CD69⁺ BM memory CD8⁺ T cells had a significantly decreased half-life compared to the PB counterparts (p=0.0027). When culturing cells with 4% O_2, no significant differences in half-lives were detected between any of the memory T cell subsets and half-life of BM memory T cells were increased compared to the ones obtained with 21% O_2.

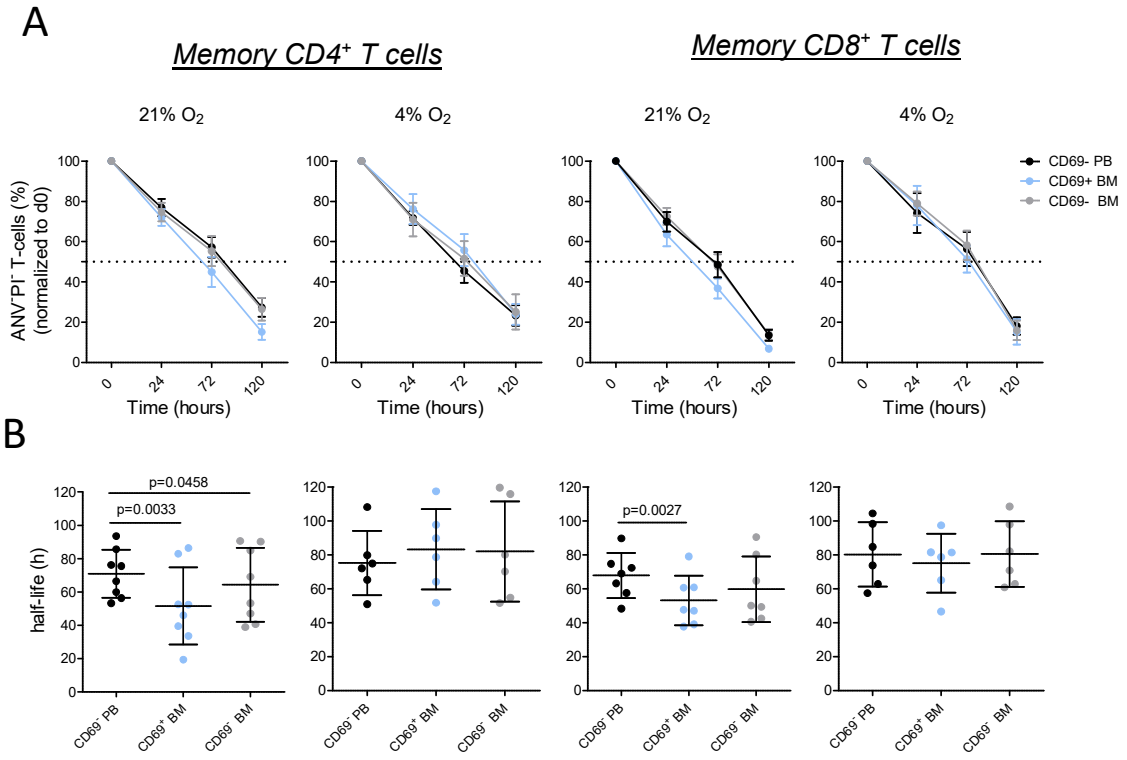


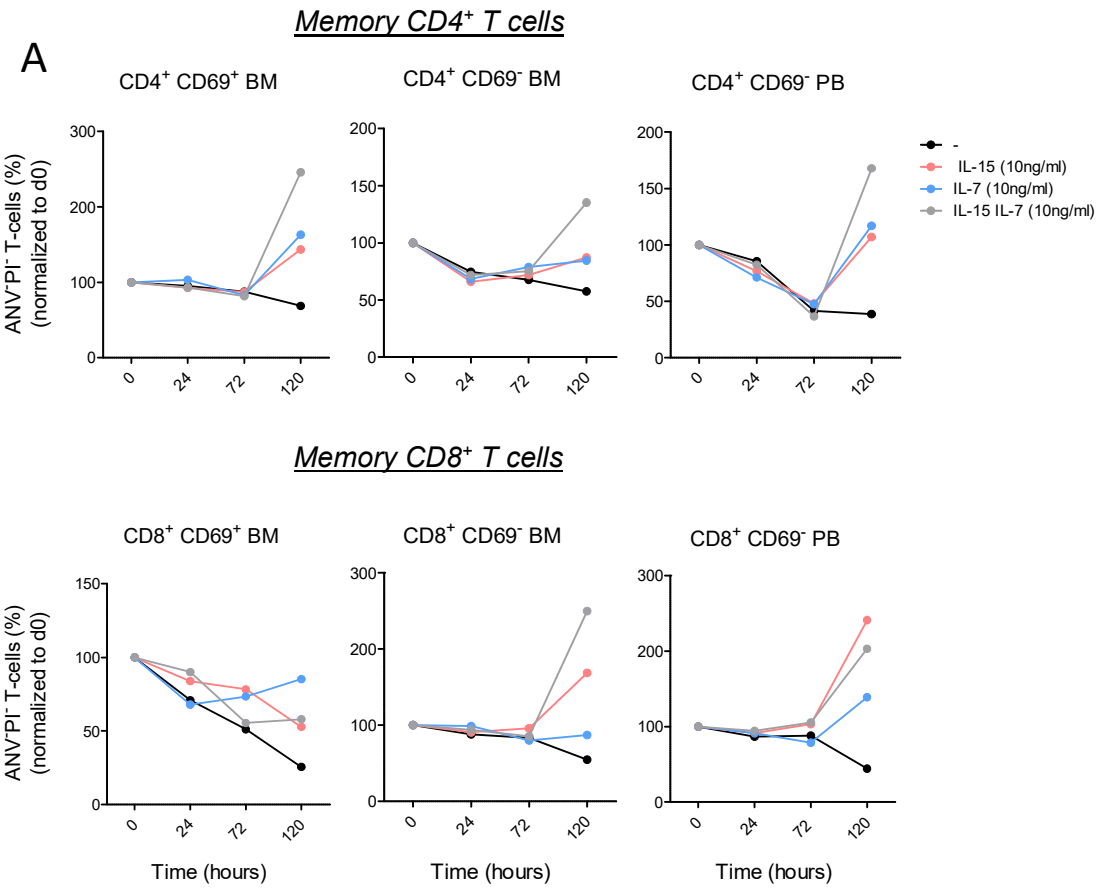
Figure 3-1: Different *ex vivo* PB and BM cell survival. *Ex vivo* CD69⁺ and CD69⁻ memory CD4⁺ and CD8⁺ T cells were isolated from paired blood and BM samples (n=6). Cells were cultured in RPMI supplemented with 5% human AB serum. (A) Cell viability was assessed by flow cytometry at the indicated time points by annexin V (ANV) and propidium iodide (PI) staining and frequencies of surviving cells were normalized to the numbers of viable cells counted at day 0. (B) Half-life was calculated for each individual donor and plotted for the different cell subsets.

3.1.2 Determination of IL-7/IL-15 concentration to maintain resting, non-proliferating memory T cells

Several studies have already demonstrated the involvement of IL-7 and IL-15 in the survival of memory $CD8^+$ T cells [144,145] and memory $CD4^+$ T cells, although with controversial data for the latter [144,147]. Previous studies from our group showed that BM memory T cells are residing in specific survival niches, where they rest in terms of proliferation, transcription and migration [59,135]. In order to determine the appropriate working concentration to maintain memory T cell survival without inducing proliferation, I tested an array of concentrations of both IL-7 and IL-15 (0.1/1/10 ng/mL) and measured the survival rates at indicated time points following the protocol as described before.

High concentrations of either cytokines (10 ng/mL) induced proliferation of PB and BM memory $CD4^+$ and $CD8^+$ memory T cells, identified by the appearance of blasting cells and the increase of cell numbers at day 5 of culture. Cell numbers at day 5 of culture increased by 1.5- to 3-fold compared to the number of cells plated at day 0 (Figure 3.2A). On the other hand, when using 0.1-1 ng/mL of either cytokines, none of the effects mentioned above was detected, suggesting that cytokine concentrations below 1 ng/mL maintain memory T cells in a resting state. Survival of memory $CD4^+$ T cells was enhanced when cultured with 1 ng/mL compared to 0.1 ng/mL, with some variations in $CD8^+$ memory T cells. Interestingly, we detected no significant improvement in memory $CD4^+$ T cell survival when adding both IL-7 and IL-15 to the cell cultures compared to the effect of each cytokine alone (Figure 3.2B).

Finally, to control whether proliferation was induced in the presence of 1ng/ml of cytokines, I measured the expression of Ki-67 in BM memory T cells at day 3 of culture with or without IL-7 and IL-15 at 1 ng/mL. In all analyzed conditions (-, IL-7 at 1 ng/mL or IL-15 at 1 ng/mL), the frequency of $Ki\text{-}67^+$ cells remained below 1%, and no increased proliferation was detected in the conditions where cytokines were added at a concentration of 1 ng/mL (Figure 3.2C). Taking all this information into account, I chose 1 ng/mL as the concentration to be used for further survival experiments, as it was able to maintain survival of all memory T cell subsets to a greater extent than 0.1 ng/mL, keeping cells in a resting state, with no signs of proliferation.


A
Memory CD4+ T cells
CD4+ CD69+ BM
CD4+ CD69- BM
CD4+ CD69- PB
ANV-PI- T-cells (%) (normalized to d0)
-
IL-15 (10ng/ml)
IL-7 (10ng/ml)
IL-15 IL-7 (10ng/ml)
Memory CD8+ T cells
CD8+ CD69+ BM
CD8+ CD69- BM
CD8+ CD69- PB
Time (hours)

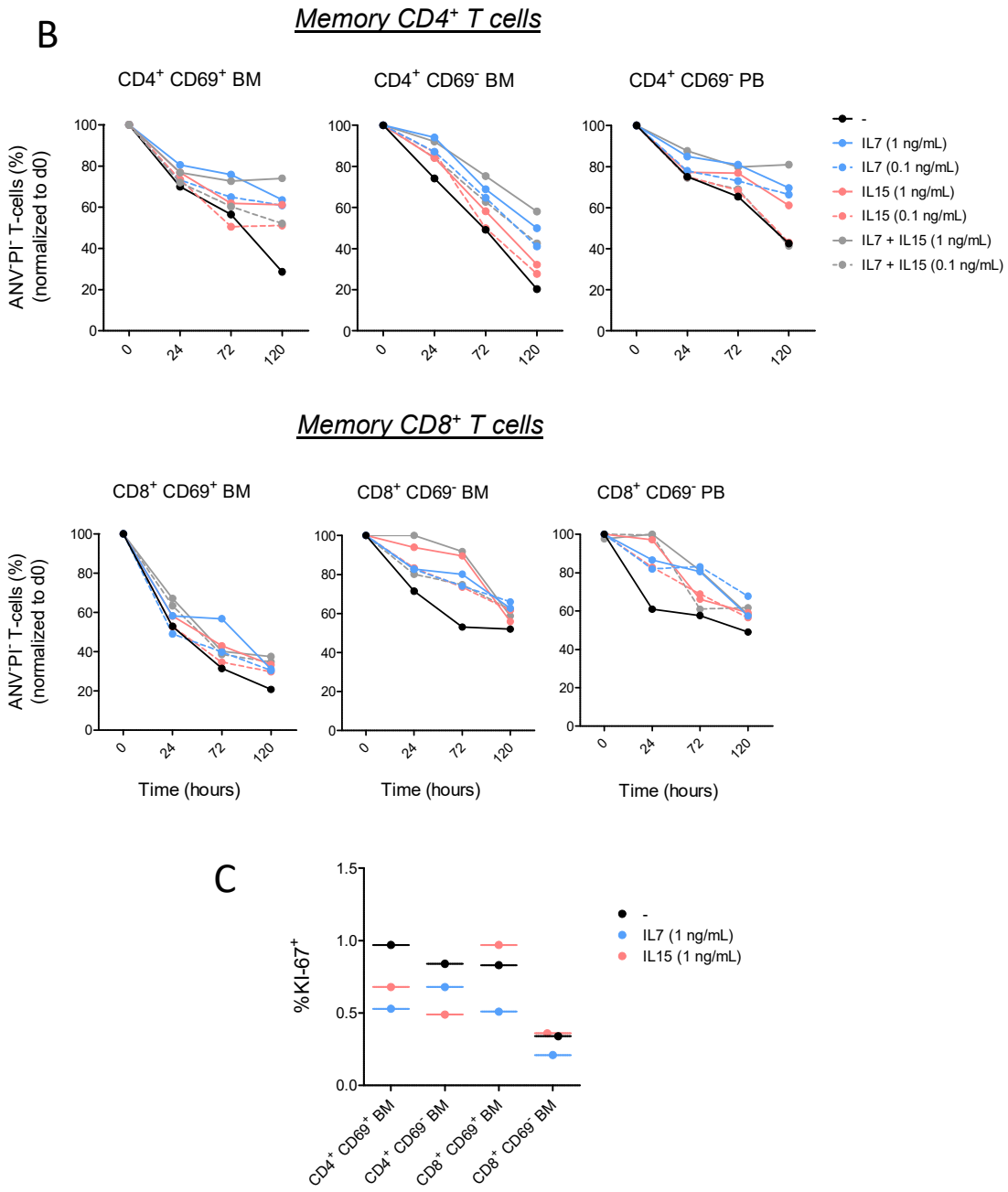


Figure 3-2: Determination of concentrations of IL-7 and IL-15 that support memory T cell survival without induction of proliferation. CD69$^+$ and CD69$^-$ memory CD4$^+$ and CD8$^+$ T cells were isolated from BM samples. Cells were cultured under 21% O_2 in RPMI supplemented with 5% human AB serum (-) or in medium supplemented with IL-7 and IL-15 at 0.1-1-10 ng/mL. Cell viability was assessed by flow cytometry at the indicated time points by annexin V (ANV) and propidium iodide (PI) staining and frequencies of surviving cells were normalized to the numbers of viable cells counted at day 0. (A) Test of IL-7 and IL-15 at 10 ng/mL. (B) Test of IL-7 and IL-15 at 0.1-10 ng/mL. (C) Ki-67 expression was measured in memory T cells at day 3 of culture by intracellular staining.

3.1.3 IL-7 and IL-15 but no other cytokines tested support memory T cell survival

IL-7 and IL-15 showed to have a clear effect in sustaining memory T cell survival. In addition to that, data supporting the role of other cytokines, such as IL-33, TGFβ and TNF, which have been implicated in the generation of tissue resident memory T cells, on the maintenance of memory T cells is sparse [92]. In order to identify possible additional soluble factors involved in the maintenance of BM memory T cells, I cultured sorted memory $CD4^+$ and $CD8^+$ T cells with or without different cytokines (IL-7 andIL-15 at 1 ng/ mL; and IL-6, IL-33, TGFβ and TNFα at 10 ng/mL) and assessed their survival. Of all the tested cytokines, only IL-7 and IL-15 improved cell survival compared with medium-only controls in both, $CD4^+$ and $CD8^+$ memory T cells (Figure 3.3). Less than 20% of memory T cells cultured in medium-only conditions (-) were alive after 5 days of culture. The addition IL-7 and IL-15 to the cultures maintained ~40% of $CD4^+CD45RA^-CD69^+$ and ~55% of $CD4^+CD45RA^-CD69^-$ at day 5 of culture, with IL-7 having a slightly better effect than IL-15 in both cell populations (Figure 3.3A). When looking at CD8+ memory T cells, both cytokines also kept ~50% of $CD8^+CD45RA^-CD69^+$ and ~40% of $CD4^+CD45RA^-CD69^-$ alive at day 5 of culture, with IL-15 being more effective in maintaining cell survival in this case (Figure 3.3B).

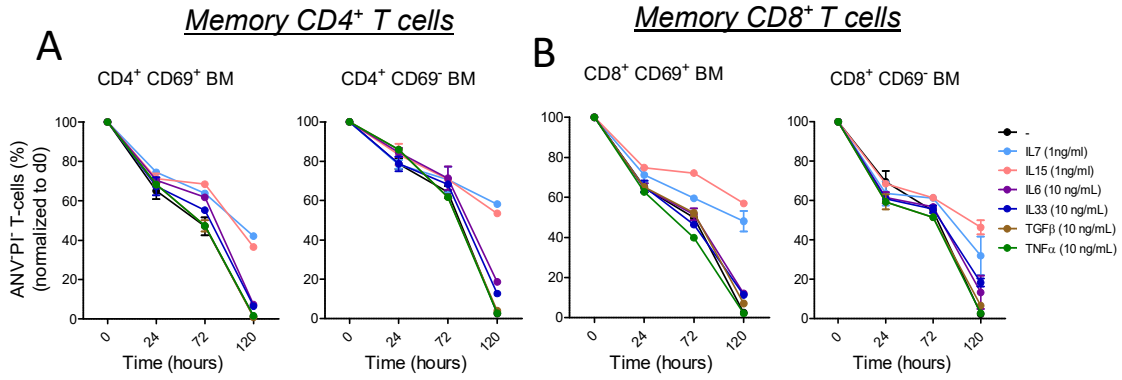


Figure 3-3: IL-7 and IL-15 but no other cytokines tested support memory T cell survival. $CD69^+$ and $CD69^-$ memory $CD4^+$ and $CD8^+$ T cells were isolated from BM samples. Cells were cultured under 21% O_2 in RPMI supplemented with 5% human AB serum (-) or in medium supplemented with indicated cytokines and concentrations. Cell viability was assessed by flow cytometry at the indicated time points by annexin V (ANV) and propidium iodide (PI) staining and frequencies of surviving cells were normalized to the numbers of viable cells counted at day 0. (A) Survival curves for BM memory $CD4^+$ T cells. (B) Survival curves for BM memory $CD8^+$ T cells.

3.1.4 IL-7 and IL-15 partially but significantly increase the survival of PB/BM memory T cells

After testing different cytokines and selecting the right working concentrations of IL-7 and IL-15, I performed a more extensive analysis of the effect of the cytokines in cell survival using cells from 6 donors (2 males and 4 females, age 70 ± 10). I cultured memory T cells from both PB and BM from the same donor under 21% O_2 with or without the indicated cytokines at a concentration of 1 ng/mL. As shown above (Figure 3.1), under these oxygen conditions both PB memory $CD4^+$ and $CD8^+$ T cells survived better than the BM counterparts in the absence of any additional stimulus (Figure 3.4).In $CD4^+$ memory T cells, IL-7 had a significant effect on cell survival, significantly increasing cell survival of all cells at day 5 of culture; from ~20% of cells alive in medium-only conditions to > 50% alive when the cytokine was present (p=0.0019 in BM $CD4^+CD69^+$, p=0.0052 in BM $CD4^+CD69^-$ and p< 0.0001 in PB $CD4^+CD69^-$). IL-15 also improved cell survival, but to a lesser extent, maintaining from 40 to 48% of cells alive at day 5 and reaching only significance at day 5 in BM $CD4^+CD69^+$ (p=0.017) and PB $CD4^+CD69^-$ (p=0.002) cells (Figure 3.4A). I found much more variances between donors when analyzing the survival of $CD8^+$ memory T cells. However, both cytokines also increased $CD8^+$ memory T cell survival in a similar way. In particular, survival of BM $CD8^+CD69^+$ T cells was significantly increased at day 5 of culture (p=0.0182). Moreover, IL-7 and IL-15 significantly supported the survival of PB $CD8^+CD69^-$ cells at day 5 of culture (p=0.002 for IL-7 and p=0.001 for IL-15) (Figure 3.4B). Altogether, these results suggest that IL-7 and IL-15 are able to maintain the survival of memory T cells, significantly increasing the numbers of live cells at days 3 or 5 of culture. However, I could not maintain more than 65% of the cells alive in any of the assays performed; suggesting that they may be additional factors involved in the long-term maintenance of memory T cells.

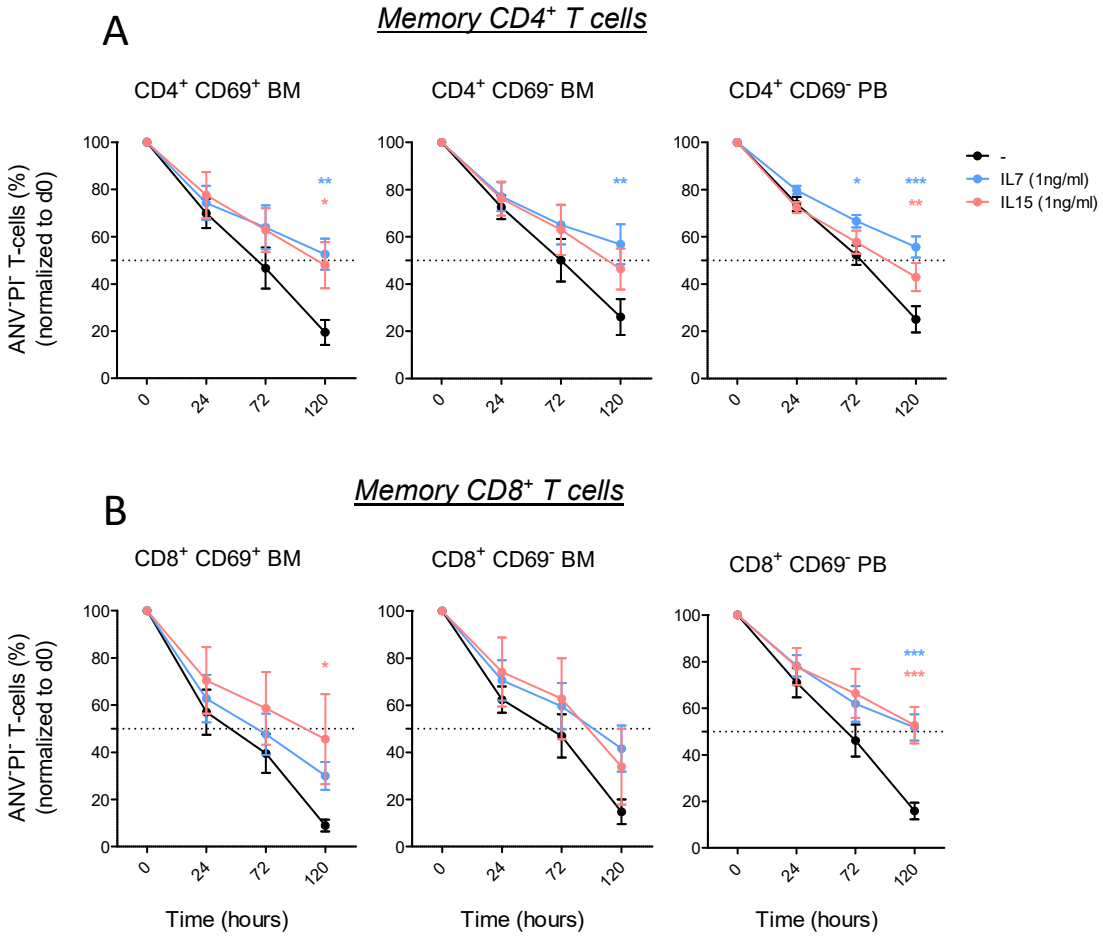


Figure 3-4: IL-7 and IL-15 significantly increase survival of memory T cells. *Ex vivo* CD69$^+$ and CD69$^-$ memory CD4$^+$ and CD8$^+$ T cells were isolated from BM samples. Cells were cultured under 21% O_2 in RPMI supplemented with 5% human AB serum (-) or in medium supplemented with IL-7 or IL-15 at 1 ng/mL. Cell viability was assessed by flow cytometry at the indicated time points by annexin V (ANV) and propidium iodide (PI) staining and frequencies of surviving cells were normalized to the numbers of viable cells counted at day 0. (A) Survival curves for BM memory CD4$^+$ T cells. (B) Survival curves for BM memory CD8$^+$ T cells. Statistical differences were calculated by 2-WAY ANOVA with Turkey correction ($p<0.0332$ (*), $p<0.0021$ (**), $p<0.0002$ (***), $p<0.00001$ (****)).

3.1.5 Stromal cells maintain memory T cell survival similar to cytokines

Apart from the soluble factors, cell-cell contacts between T cells and stromal cells are also thought to be important for the maintenance of memory T cells. Molecules expressed by stromal cells, such as VCAM-1 and collagens I, II and XI were shown to interact with

specific receptors expressed in memory T cells and could contribute to the maintenance of memory T cells. To test the role of stromal cells in memory T cell survival, I used a human bone marrow stromal cell line (HS5) and compared its effect on memory T cell survival with the one exerted by the cytokines. Publicly available transcriptomes from this cell line (http://www.ncbi.nlm.niih.gov/geo) show that the HS5 cells do not express IL-7 and express only little amounts of IL-15, whereas they express high amounts of VCAM-1 and collagens in their surface. Moreover, analysis of supernatants of stromal cell co-cultured with and without memory T cells by Luminex beads revealed no detectable levels of IL-7 and IL-15 (data generated by Jessica Dysarz during her Bachelor´s Thesis). As the human BM oxygen levels are approximately 6% [159], I performed the following experiments using 4% O_2, in order to mimic as closely as possible the BM environment.

I analyzed cells of 6 donors (3 males and 3 females, age 66 ± 8) and determined the survival of PB and BM memory T cells without any stimulus (-), in the presence of IL-7 and IL-15, or when co-cultured with HS5 stromal cells. As showed before (Figure 3.1), when culturing memory T cells under reduced levels of oxygen (4% O_2), BM memory T cells survive better than their PB counterparts when no additional stimulus is present (Figure 3.5). For memory $CD4^+$ T cells, IL-7 and IL-15 had similar effects than the ones described in section 3.1.4. When memory T cells were cultured without any additional stimulus (-), ~20% of the cells were alive at day 5 of culture, whereas IL-7 and IL-15 were able to maintain ~50% of cells alive at day 5. Co-culture of $CD4^+$ memory T cells with HS5 resulted in a significant improvement of survival, keeping from 50 to 62% of memory T cells alive at day 5 of culture. Cell survival was significantly improved in BM $CD4^+CD69^+$ ($p=0.009$ (day 3) and $p<0.0001$ (day 5), BM $CD4^+CD69^-$ ($p=0.0137$ (day 3) and $p<0.0001$ (day 5) as well as PB $CD4^+CD69^-$ ($p=0.0006$ (day 1), $p<0.0001$ (day 3) and $p<0.0001$ (day 5) (Figure 3.5A). When analyzing memory $CD8^+$ T cells, I could also detect a similar effect of the cytokines as described in section 3.1.4, with IL-15 showing a greater role in the maintenance of memory $CD8^+$ T cells compared with IL-7. Co-culture of memory $CD8^+$ T cells with HS5 cells increased the survival of memory T cells from ~20% to 50-60% at day 5 of culture. The numbers of viable cells were increased in BM $CD8^+CD69^+$ ($p=0.001$ (day 3) and $p<0.0001$ (day 5), BM $CD8^+CD69^-$ ($p<0.0001$ (day 5) as well as PB $CD8^+CD69^-$ ($p=0.0059$ (day 1), $p<0.0001$ (day 3) and $p<0.0001$ (day 5) (Figure 3.5B). Altogether, the co-culture of memory T cells with a bone marrow stromal cell line maintains the survival of the cells, to the same or better extent than IL-7 and IL-15. However, neither

cytokines nor stromal cells are sufficient by themselves to maintain memory T cells long-term, as they cannot keep all cells alive after 5 days of culture (Figure 3.5).

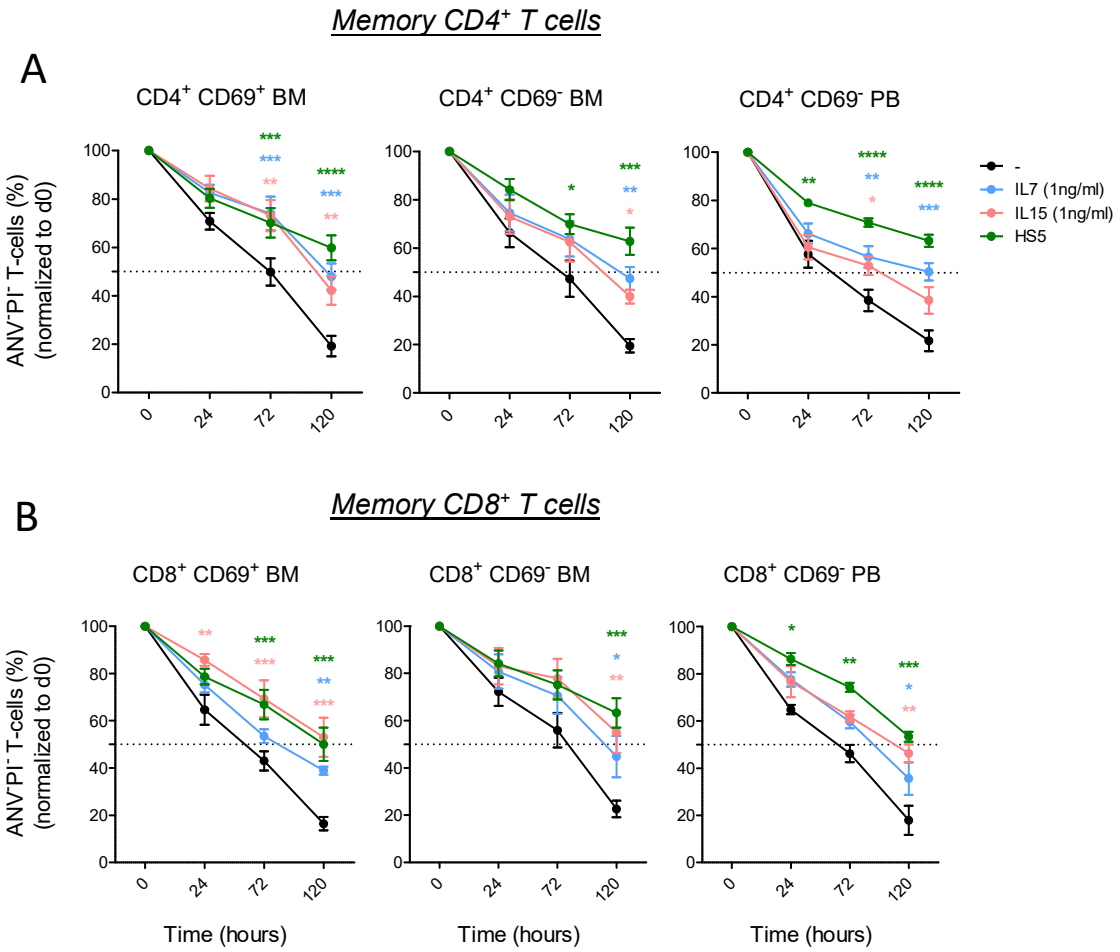


Figure 3-5: IL-7 / IL-15 and stromal cells are involved in the maintenance of memory T cells survival. *Ex vivo* $CD69^+$ and $CD69^-$ memory $CD4^+$ and $CD8^+$ T cells were isolated from BM samples. Cells were cultured under 4% O_2 in RPMI supplemented with 5% human AB serum (-), in medium supplemented with IL-7 or IL-15 at 1 ng/mL or co-cultured with HS5. Cell viability was assessed by flow cytometry at the indicated time points by annexin V (ANV) and propidium iodide (PI) staining and frequencies of surviving cells were normalized to the numbers of viable cells counted at day 0. (A) Survival curves for BM memory $CD4^+$ T cells. (B) Survival curves for BM memory $CD8^+$ T cells. Statistical differences were calculated by 2-WAY ANOVA with Turkey correction ($p<0.0332$ (*), $p<0.0021$ (**), $p<0.0002$ (***), $p<0.00001$ (****)).

Next, I analyzed the expression of the IL-7Rα (CD127) as marker of IL-7 signaling. It has been shown, that exposure to IL-7 decreases cell-surface expression of CD127 in $CD4^+$ [183] and memory $CD8^+$ T cells within 24-48 h of culture [184]. Therefore, I aimed to know whether the culture of our memory T cells, either with cytokines or the stromal cells, induced a down-regulation of CD127.

I could detect a rapid down-regulation of CD127 expression in all memory T cell populations when cultured with IL-7, compared to the non-stimulated (-) samples. This down-regulation was already visible 24h after the start of the cultures and was maintained during all the analyzed time-points, suggesting the continuous action of IL-7. Moreover, IL-15 also induced a down-regulation of CD127 in BM CD69$^-$ and PB CD69$^-$, for both CD4$^+$ and CD8$^+$ memory T cells (Figure 3.6), albeit to a lower degree. This data further reinforces the hypothesis that stromal cells support memory T cell survival via different mechanisms than cytokine secretion (IL-7 and IL-15), as the co-culture of memory T cells with HS5 cells did not induce down-regulation of CD127 surface expression.

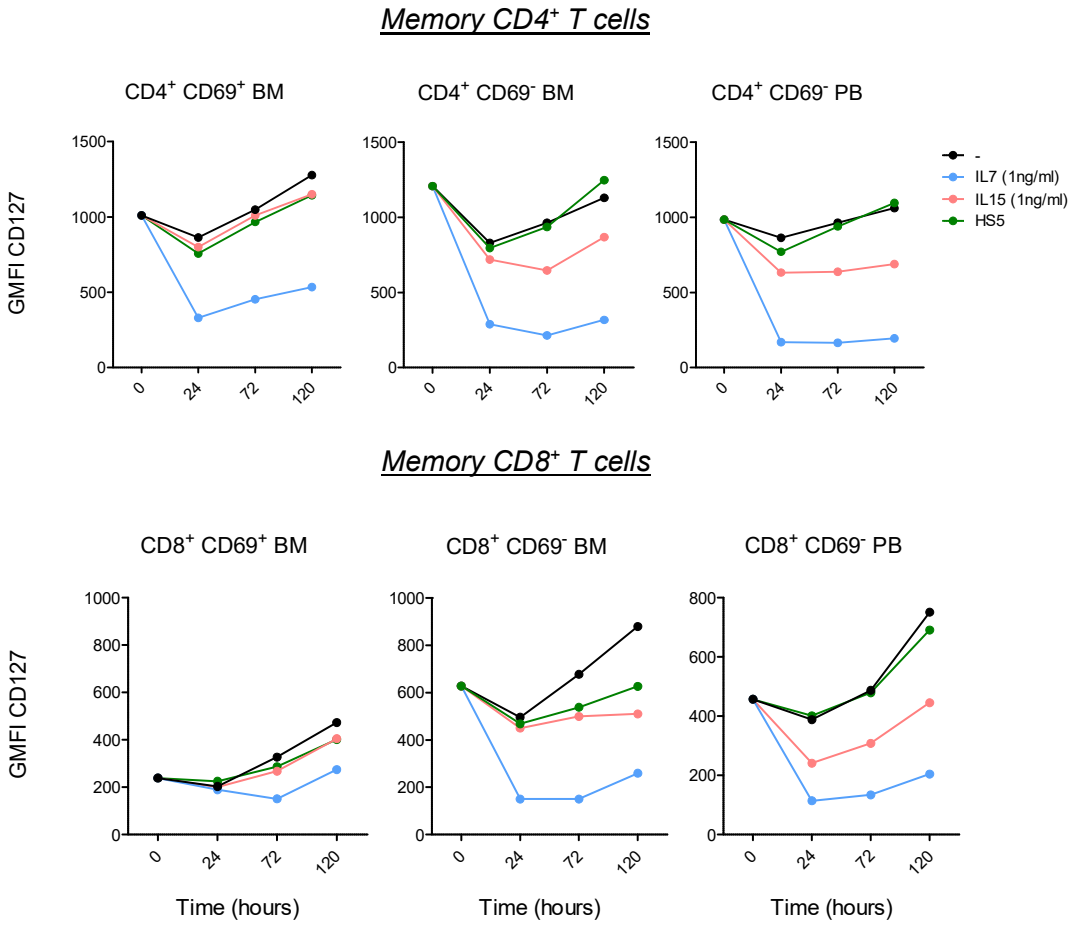


Figure 3-6: IL-7 and IL-15, but not co-culture with stromal cells down-regulate CD127 expression on memory T cell surface. *Ex vivo* CD69$^+$ and CD69$^-$ memory CD4$^+$ and CD8$^+$ T cells were isolated from BM samples. Cells were cultured under 4% O_2 in RPMI supplemented with 5% human AB serum (-), in medium supplemented with IL-7 or IL-15 at 1 ng/mL or co-cultured with the human BM stromal cell line HS5. CD127 surface expression was analyzed on live cells at different timepoints.

3.1.6 Stromal cells mediate memory T cell survival via contact-dependent mechanisms

The HS5 human bone marrow stromal cell line does not express IL-7 at an mRNA level and does not produce high levels of either, IL-7 and IL-15 proteins, as they were not detected in the supernatants by Luminex analysis (performed by Jessica Dysarz). Other soluble factors produced by stromal cells as well as cell-cell interactions could be responsible for the maintenance of memory T cells. To determine whether the effect on cell survival performed by HS5 cells was cell-contact dependent or not, I designed a transwell (TW) assay (performed by Jessica Dysarz) in which memory T cells were plated on the top of a membrane which was placed onto a well seeded with stromal cells. This assay allows the memory T cells to be in contact with the soluble factors secreted by the stromal cell line without having direct contact with it. The analysis of viable cell numbers at day 3 of culture, comparing memory T cells without any stimulus (-), in direct contact with HS5 cells or in transwells (TW) revealed that the survival effect produced by stromal cells is contact-dependent (Figure 3.7A). In all donors analyzed (n=3), we could reproducibility detect an increased frequency of viable memory T cells (40-60%) when cultured in direct contact with HS5 , whereas the survival of BM memory T cells cultured in the transwells remained similar to the non-stimulated ones (10-20%).

In addition, we tested the capacity of HS5 supernatants to support the survival of BM memory T cells (study performed by Kathrin Stilz during her Master´s Thesis). For that, we took the supernatant of 1 day cultured stromal cells and added it to the memory T cell cultures. As shown before, BM memory $CD4^+$ and $CD8^+$ T cell directly co-cultured with HS5 cells had significantly increased survival (Figure 3.7B). The addition of stromal cell supernatant (SBNT) to memory T cell cultures resulted only in a minor increase of surviving cells, reinforcing the idea that BM stromal cells exert their role on the maintenance of memory T cells mainly via cell-cell interactions.

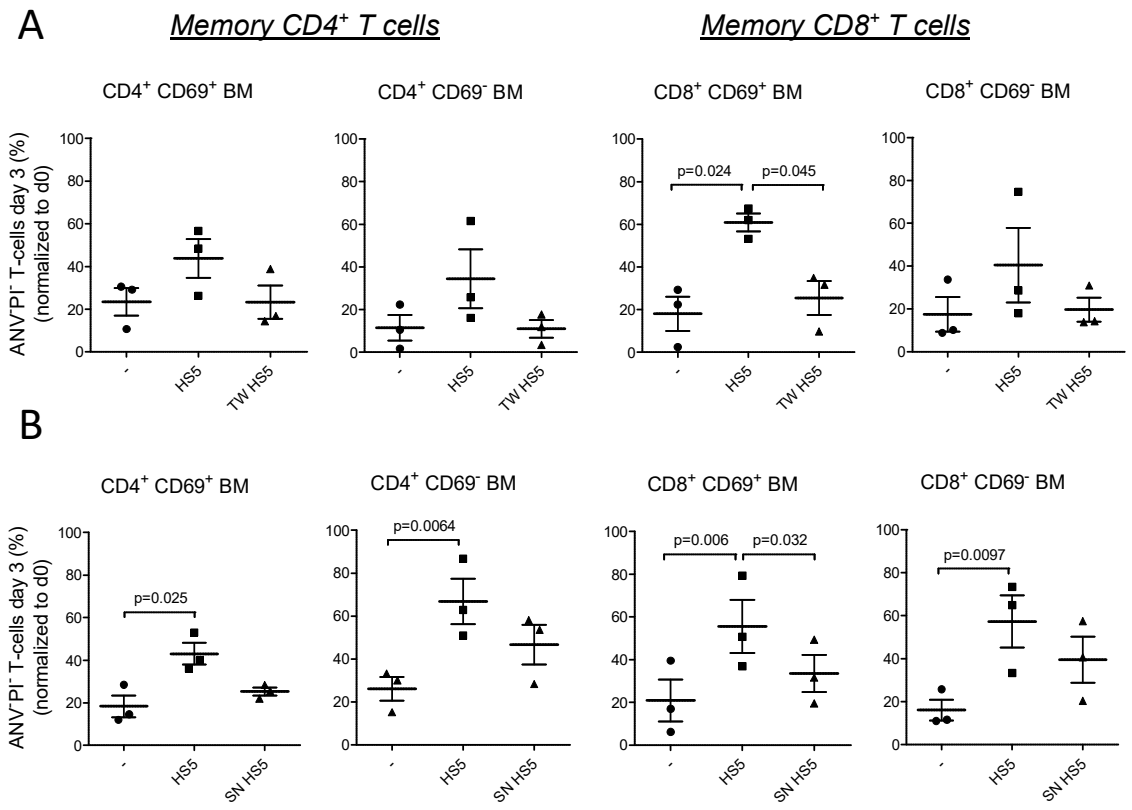


Figure 3-7: Cell-contact dependency of memory T cells with BM stromal cells. *Ex vivo* isolated CD69$^+$ and CD69$^-$ memory CD4$^+$ and CD8$^+$ T cells of BM were were cultured under 4% O_2 in RPMI supplemented with 5% human AB serum (-) or in co-culture with stromal cells in different conditions. (A) Memory T cells were cultured directly with HS5 stromal cells or in transwell (TW). (B) Memory T cells were cultured in direct contact with HS5 cells or with HS5 supernatants (SBNT). Cell viability was assessed by flow cytometry at the indicated time-points by annexin V (ANV) and propidium iodide (PI) staining and frequencies of surviving cells were normalized to the numbers of viable cells counted at day 0.

3.1.7 Combination of cytokines and HS5 cells induce memory T cell proliferation

Histological analysis of memory CD4$^+$ T cells in the murine BM have revealed direct contact of these cells with VCAM$^+$ IL-7 producing stromal cells [77], suggesting both factors, cell-cell contact and cytokine signaling, are involved in the maintenance of memory T cells. Moreover, when culturing memory T cells either with cytokines or stromal cells, I could only achieve a partial effect in the survival of memory T cells. Therefore, I decided to combine both factors in order to investigate whether they could have synergistic roles in the long-term maintenance of memory T cells. I cultured BM memory T cells in the

presence of HS5 cells and cytokines, and measured survival (Figure 3.8). In all BM memory T cell populations, I detected an increase of cell numbers from day 3 to day 5 when cells were cultured with HS5 cells and cytokines, suggesting that the levels of cytokines used together with HS5 were inducing T cell proliferation (Figure 3.8A).Thus, I titrated the cytokines from 1 ng/mL to 0.001 ng/mL in order to see whether with lower cytokine concentrations memory T cells stayed in a quiescent state. The addition of IL-7 and IL-15 at concentrations of 1-0.1 ng/mL with the stromal cell line induced an increase on the cell numbers of memory T cells at day 5 compared with day 3, indicating proliferation. Concentrations of 0.01 ng/mL did not induce proliferation, but also did not show a significant improvement in cell survival when comparing with memory T cells co-cultured with HS5 alone (Figure 3.8B). Taking this information into account, until now, our in vitro system does not allow us to fully mimic the conditions found in the BM environment, where memory T cells are maintained in a resting state [59].

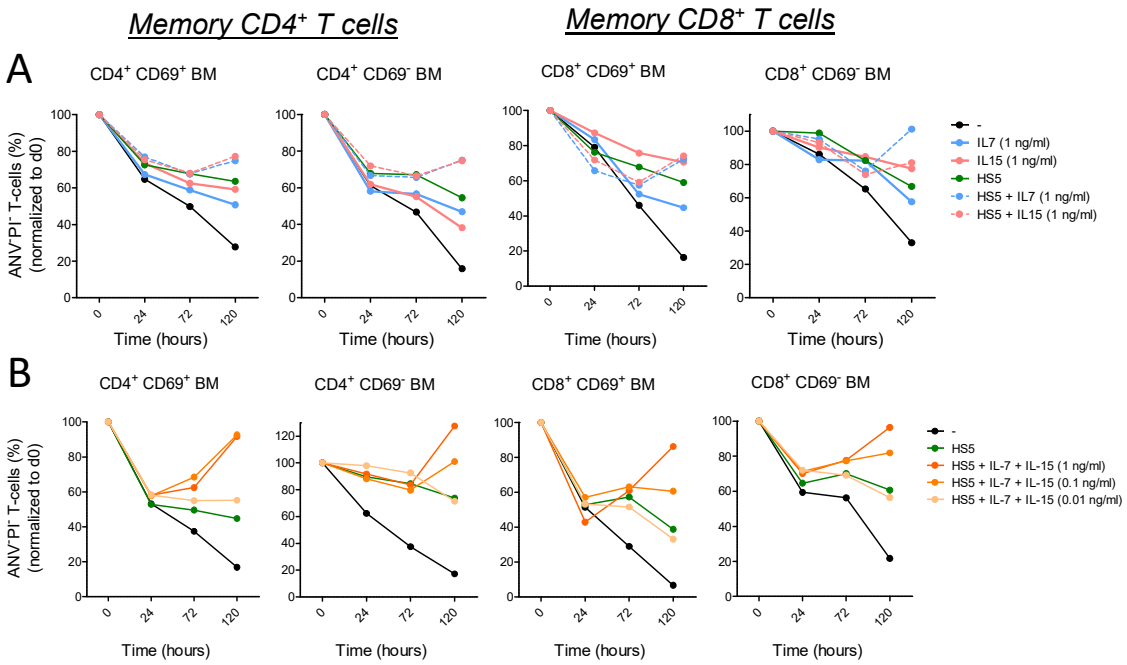


Figure 3-8: No synergistic effects of cytokines and stromal cells in the maintenance of memory T cells survival. $CD69^+$ and $CD69^-$ memory $CD4^+$ and $CD8^+$ T cells were isolated from BM samples. Cells were cultured under 4% O_2 in RPMI supplemented with 5% human AB serum (-), or in co-culture with HS5 stromal cells with and without cytokines. Cell viability was assessed by flow cytometry at the indicated time-points by annexin V (ANV) and propidium iodide (PI) staining and frequencies of surviving cells were normalized to the numbers of viable cells counted at day 0. (A) Memory T cells were cultured with HS5 plus IL-7 or IL-15 at 1 ng/mL. (B) Memory T cells were cultured with HS5 stromal cells and different concentrations of IL-7plus IL-15 (0.01-0.1 and 1 ng/mL).

3.1.8 Cytokines and HS5cells support memory T cell survival via different molecular mechanisms

So far, results obtained in our studies suggested that either cytokines or stromal cell contact contribute to the survival of non-proliferating memory T cells via different pathways. The co-culture of memory T cells with HS5 cells did not induce a down-regulation of CD127 (Figure 3.6) and the analysis of survival when using the transwell and the stromal cell supernatant indicated the contact dependency between stromal cells and memory T cells (Figure 3.7).

It is known, that the receptors for the common γ-chain cytokines (including IL-7 and IL-15) are involved in the regulation of circulating naïve and memory T cell homeostasis [145], inducing the expression of anti-apoptotic factors (BCL-2 and BCL-XL). Moreover, BIM is a probable candidate for cell death inducer after IL-7 or IL-15 withdrawal from memory T cells [171]. However, the survival pathways involved in memory T cell maintenance and the role of these factors in activating or inhibiting the different pro- and anti-apoptotic molecules remains still unknown. In order to better understand which molecular factors are important for memory T cell survival and how are they modulated by IL-7, IL-15 or cell contact with stromal cells, we measured the expression of two pro-apoptotic (BIM and NOXA) and two anti-apoptotic (BCL-2 and MCL-1) factors in memory T cells under different conditions at day 3 of in vitro culture.

I detected a significant increase in the anti-apoptotic molecule BCL-2 expression in the presence of both IL-7 and IL-15, but not when cells were co-cultured with the stromal cell line HS5. On average, the increase on the mean fluorescence intensity of BCL-2 was 0.5-fold ($p<0.0001$) with IL-7 and 0.3-fold ($p=0.0005$) with IL-15 in the BM CD4 $CD69^+$ memory T cells, 0.46-fold ($p<0.0001$) with IL-7 and 0.26-fold ($p=0.00073$) with IL-15 in the BM CD4 $CD69^-$ memory T cells and 0.48-fold ($p<0.0001$) with IL-7 and 0.17-fold ($p=0.022$) with IL-15 in the PB CD4 $CD69^-$ memory T cells. In all cases IL-7 increased BCL-2 to a greater extent than IL-15, correlating with the increased survival of memory $CD4^+$ T cells when cultured with IL-7 compared to IL-15 (Figures 3.4 and 3.5). I did not observe any significant differences in the mean fluorescence intensity of the pro-apoptotic molecule BIM in any of the conditions analyzed. In consequence, the ratio of BCL-2 to BIM, an important parameter determining death or survival of the cells, was significantly increased in the memory T cells in the presence of both cytokines but not upon contact with HS5 (Figure 3.9A).

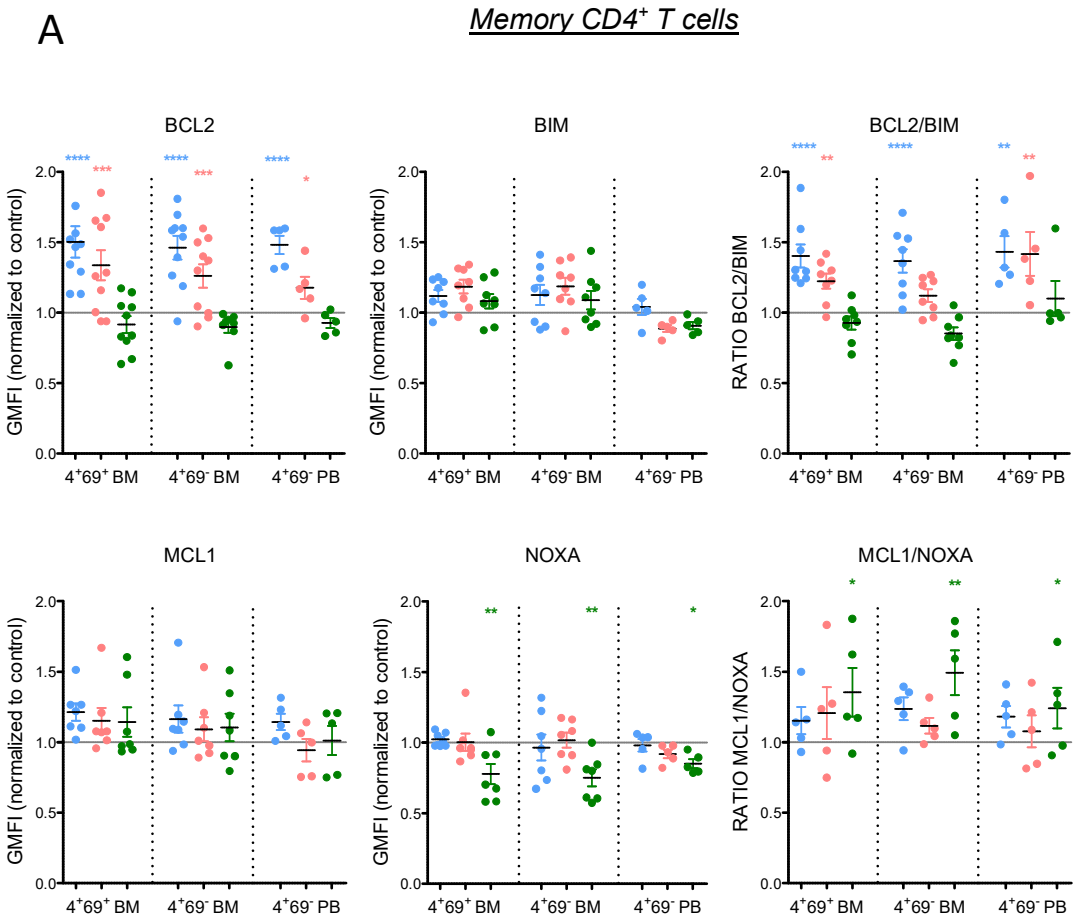
On the other hand, the mean fluorescence intensity of the pro-apoptotic protein NOXA was significantly decreased when $CD4^+$ memory T cells were co-cultured with stromal cells but not in the presence of both cytokines. On average, the decrease in the mean fluorescence intensity of NOXA was 0.23-fold (p=0.001) in the BM CD4 $CD69^+$ T cells, 0.22-fold (p=0.0018) in the BM CD4 $CD69^-$ memory T cells and 0.15-fold (p=0.0082) in the PB CD4 $CD69^-$ memory T cells. Moreover, I did not detect any significant changes in the expression of the pro-survival molecule MCL-1. As a result, I could see a significant increase of the MCL-1/NOXA ratio in memory cells co-cultured with stromal cells, but not when cultured with either IL-7 or IL-15 (Figure 3.9B).

Within memory $CD8^+$ T cells, the increase on the mean fluorescence intensity of BCL-2 was 0.42 fold (p=0.0013) with IL-7 and 0.7-fold (p<0.0001) with IL-15 in the BM CD8 $CD69^+$ memory T cells, 0.62-fold (p<0.0001) with IL-7 and 0.62-fold (p=0.00073) with IL-15 in the BM CD8 $CD69^-$ memory T cells and 0.54-fold (p<0.0001) with IL-7 and 0.2-fold (n.s.) with IL-15 in the PB CD8 $CD69^-$ memory T cells. As before, I did not detect any significant differences in BIM expression, thus leading to a significant increase of the BCL-2/BIM ratio in all memory T cell populations analyzed when cultured with IL-7 or IL-15. The BCL-2/BIM ratio was unaffected by contact with stromal cells (Figure 3.9C).

Expression of NOXA was decreased 0.37 fold (p=0.00018) in the BM CD8 $CD69^+$ memory T cells, 0.26-fold (p=0.0016) in the BM CD8 $CD69^-$ memory T cells and 0.23-fold (p=0.002) in the PB CD8 $CD69^-$ memory T cells. MCL-1 expression was unaffected resulting in a significantly increased MCL-1/NOXA ratio in cells co-cultured with the HS5 stromal cell line.

These results suggest that whereas IL-7 and IL-15 exert their survival function through up-regulation of pro-survival factors, such as BCL-2, and therefore increasing a BCL-2/BIM ratio, memory T cell contact with stromal cells down-regulates the expression of the pro-apoptotic protein NOXA, thus increasing the MCL-1/NOXA ratio. Thus, I can conclude that cytokines and contact with stromal cells are supporting memory T cell maintenance via different pathways, having each the potential to only partially maintain the survival of memory T cells (Figure 3.5).

A


Memory CD4+ T cells
BCL2
BIM
BCL2/BIM
MCL1
NOXA
MCL1/NOXA
GMFI (normalized to control)
RATIO BCL2/BIM
RATIO MCL1/NOXA
4+69+ BM
4+69- BM
4+69- PB

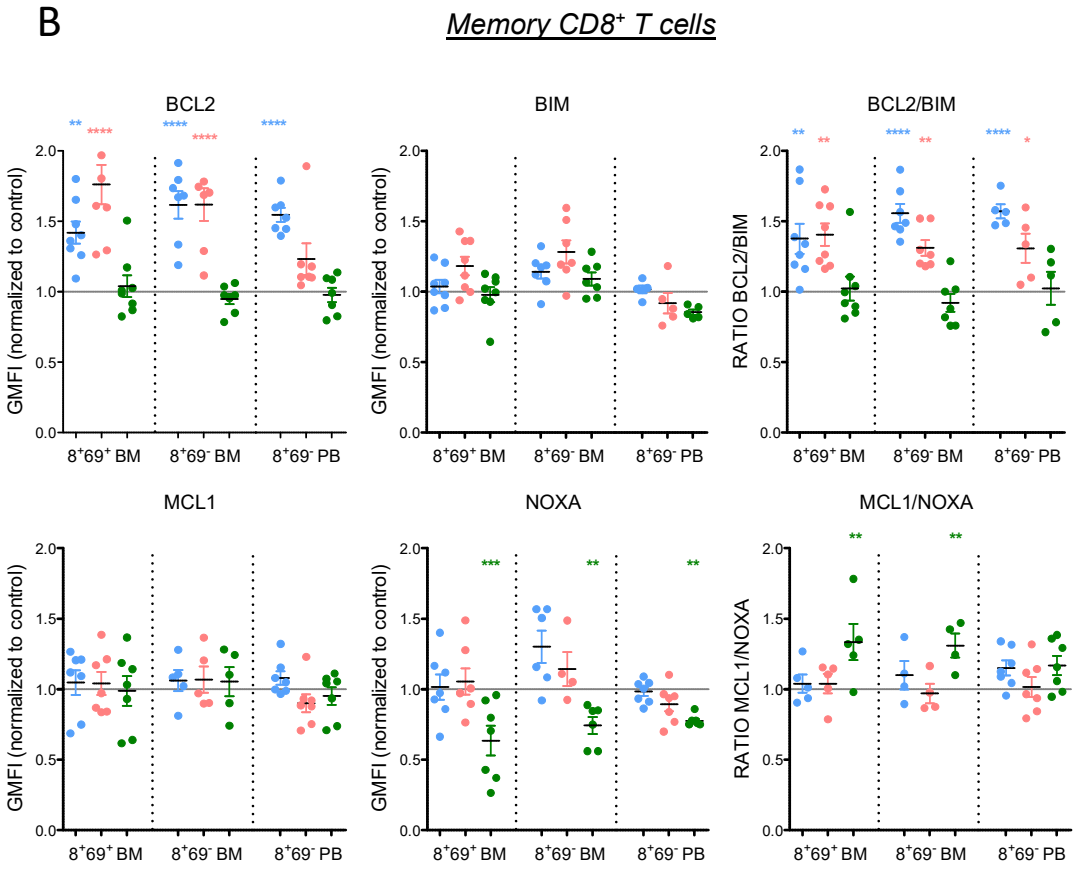


Figure 3-9: Regulation of pro- and anti-apoptotic factors in memory T cells. CD69$^+$ and CD69$^-$ memory CD4$^+$ and CD8$^+$ T cells were isolated from paired blood and bone marrow samples and. cultured under 4% O_2 in RPMI supplemented with 5% human AB serum (-) or in medium supplemented with IL-7, IL-15 or the human stromal cell line HS5. The expression of pro-/anti-apoptotic markers was determined at day 3 of culture by intracellular staining. Mean fluorescence intensity of each marker at the different tested conditions was normalized to the values detected in control samples without any stimulus (-). BCL-2/BIM and MCL-1/NOXA ratios were calculated by dividing the normalized value of one marker with the other. (A) Survival molecules expression on memory CD4$^+$ T cells (n=8). (B) Survival molecules expression on memory CD4$^+$ T cells (n=8). Statistical differences were calculated by 2-WAY ANOVA with Turkey correction ($p<0.0332$ (*), $p<0.0021$ (**), $p<0.0002$ (***), $p<0.00001$ (****)).

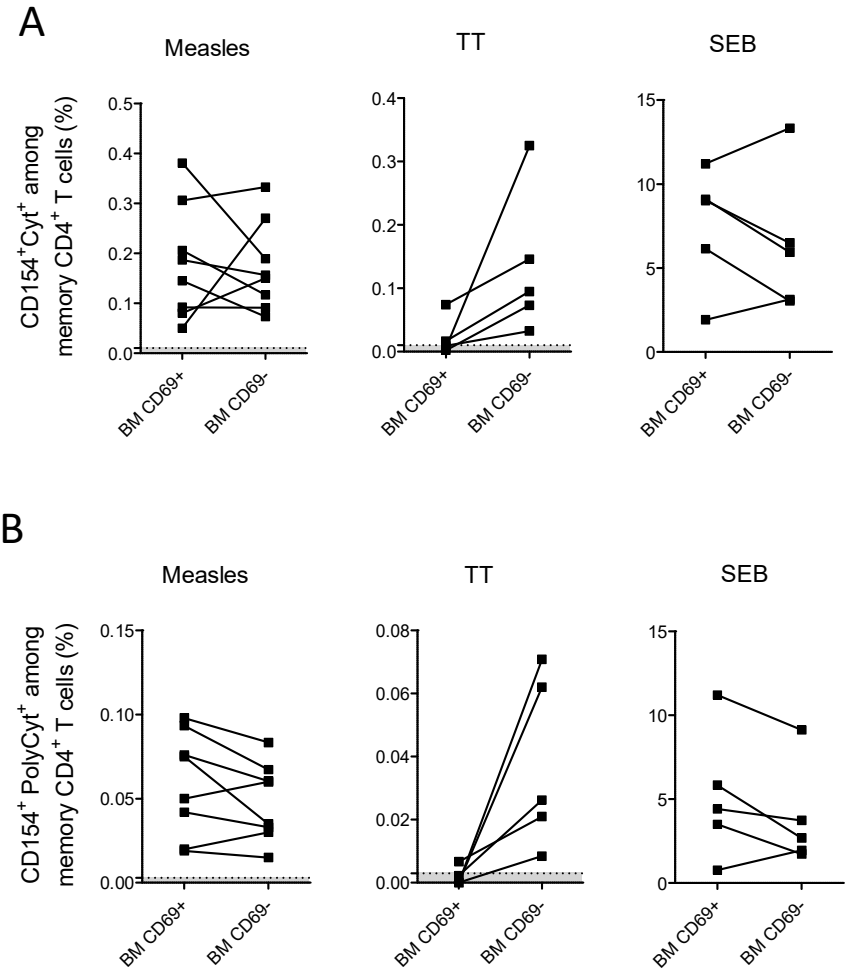
3.2 Compartmentalization of PB and BM memory T cells

3.2.1 Antigen specificities of the BM are maintained by CD69$^{+/-}$ CD4^{+} memory T cells

Previous studies performed in our group showed that the human BM is enriched for antigen-specific memory CD4^{+} T cells against long-term systemic antigens, such as measles, mumps or rubella; even when they are no longer detectable in blood circulation [59]. In order to investigate whether the enrichment of antigen-specific memory CD4^{+} T cells in BM in due to their specific accumulation in the CD69^{+} memory T cell population, or both CD69^{+} and CD69^{-} memory CD4^{+} T cells maintain these long-term specificities, I analyzed the compartmentalization of measles and TT-specific CD4^{+} memory T cells, using SEB as a positive control for the capacity of each of the populations to respond to stimulation. For that, I separated CD69^{+} and CD69^{-} memory T cells by magnetic cell separation, stained them with CFSE or efluor670 as described in material and methods, mixed and stimulated them with the corresponding antigens. In some cases, I included paired blood samples (also labeled with CFSE) when analyzing measles-specific memory CD4^{+} T cells. The magnitude of antigen-specific response was assessed by intracellular CD154, TNFα, IFNγ and IL2 staining and flow cytometric analysis. Memory CD4^{+} T cells expressing CD154 and at least one of the above-mentioned cytokines were considered specific for the tested antigens. Stimulation with aCD28 alone was performed as negative control, and background generated with this stimulation was subtracted from frequencies of cells reactive to aCD28 plus antigen before all calculations.

I did not detect any significant differences on the frequencies of measles-reactive CD69^{-} and CD69^{+} memory T cells. On average, I could detect 0.23 ± 0.18% and 0.19 ± 0.11% of measles-specific T cells and 0.056 ± 0.032% and 0.057 ± 0.029% among CD69^{-} and CD69^{+} BM memory CD4^{+} T cells, respectively (Figure 3.10A). Average frequencies of TT-specific cells differed from 0.13 ± 0.11% in the CD69^{-} to 0.021 ± 0.02% in the CD69^{+} cell subsets, showing an enrichment of TT-specific cells in the CD69^{-} compartment, albeit not statistically significant. Unspecific stimulation of BM cells with SEB resulted in similar responses in both cell subsets, with an average of 6.38 ± 4.18% in the CD69^{-} and 7.47 ± 3.59% in the CD69^{+} fractions (Figure 3.10B). The analysis of measles-specific memory CD4^{+} T cells from paired PB and BM samples corroborated previous results showing the enrichment of measles-specific memory CD4^{+} T cells in BM compared with PB[59]. 2 of the 5

donors analyzed showed frequencies of measles-reactive cells among memory $CD4^+$ T cells below the established limit of detection. In average, I detected significant differences in the percentages of measles-specific memory T cells between PB and BM, with a p=0.0122 and p= 0.0388 when compared to the $CD69^+$ and the $CD69^-$ populations respectively. As mentioned above, no differences in percentages of measles-specific memory T cells were found between $CD69^+$ and $CD69^-$ cells (Figure 3.10C).



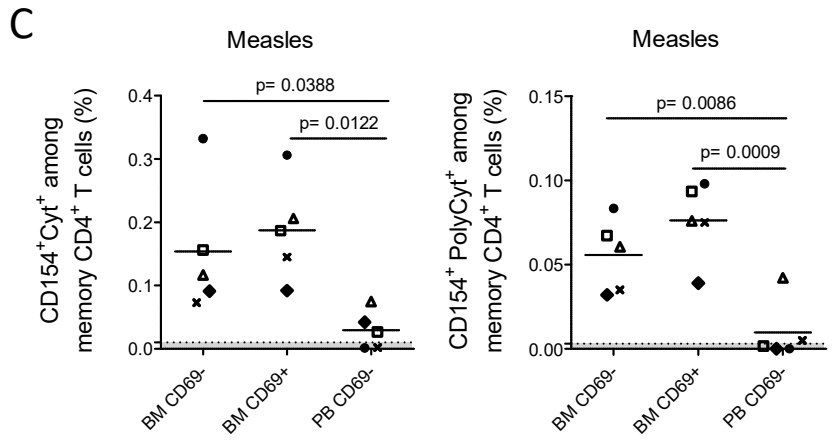
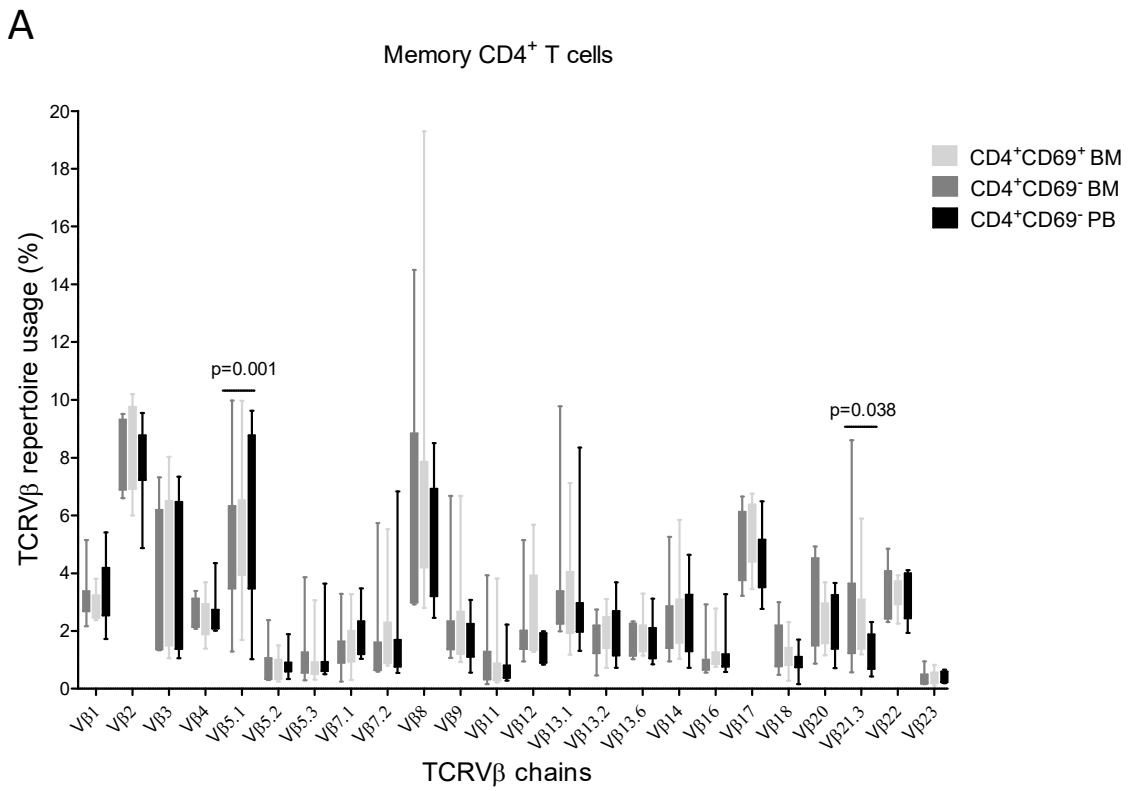


Figure 3-10: Distribution of measles and TT-specific cells in CD69$^+$ and CD69$^-$ BM memory CD4$^+$ T cells. The antigen-specific T cell response was quantified by the expression of CD154, TNFα, IFNγ and IL2. aCD28 background was subtracted from all samples. Cells positive for CD154 and expressing at least one of the analyzed cytokines were considered as antigen-specific and polyfunctional cells were calculated using Boolean gates from FlowJo software. (A) Percentages of antigen-specific T cells among memory CD4$^+$ T cells and (B) percentages of polyfunctional antigen-specific T cells. Data shown represent n=8, n=5 and n=5 for measles, TT and SEB respectively. (C) Analysis of measles-specific T cells in paired PB / BM samples. Each donor is represented by a symbol for the three different cell subsets (n=5). The reliable detection limit was set at 0.01% among memory CD4$^+$ T cells. To analyze two groups of paired samples, two-tailed Wilkoxon signed-rank test was used. For analyzing more than two groups, ANOVA analysis with Turkey correction was applied ($p<0.0332$ (*), $p<0.0021$ (**), $p<0.0002$ (***), $p<0.00001$ (****)).

3.2.2 Flow cytometric analysis reveals different TCR Vβ repertoire of PB and BM memory T cells

To determine the clonal heterogeneity of memory T cells isolated from PB versus BM, the distribution of T cell Vβ families was compared between both populations. Using a flow cytometry approach, I assessed the expression of 24 TCR Vβ chains (~70% of the Vβ TCR[181]) among CD4$^+$ and CD8$^+$ memory T cells from PB and BM. Our results showed a highly diversified TCR repertoire in memory T cells from PB and BM. All Vβ families tested were found to be present in all of the CD4$^+$ and CD8$^+$ memory T cell subsets. Moreover, frequencies of the individual TCR Vβ families in the different analyzed donors were very heterogeneous. TCR Vβ family distribution of each cell population was characterized by mean, standard deviation (SD), minimal and maximal values (Appendix tables 6.1A and 6.1B). The composition of TCR Vβ families was similar among CD69$^-$ and CD69$^+$ BM memory CD4$^+$ T cells, where no significant differences were found in any of the variants

analyzed. In contrast, I could detect a significant enrichment of Vβ5.1 in PB compared to both CD69$^{-/+}$ BM memory CD4^{+} T cells (p= 0.001) and of Vβ21.3 compared to the CD69^{-} BM population (p=0.038) (Figure 3.11). No significant differences were found among the CD8^{+} memory T cell populations, where the variation between donors was much higher (Figure 3.11).



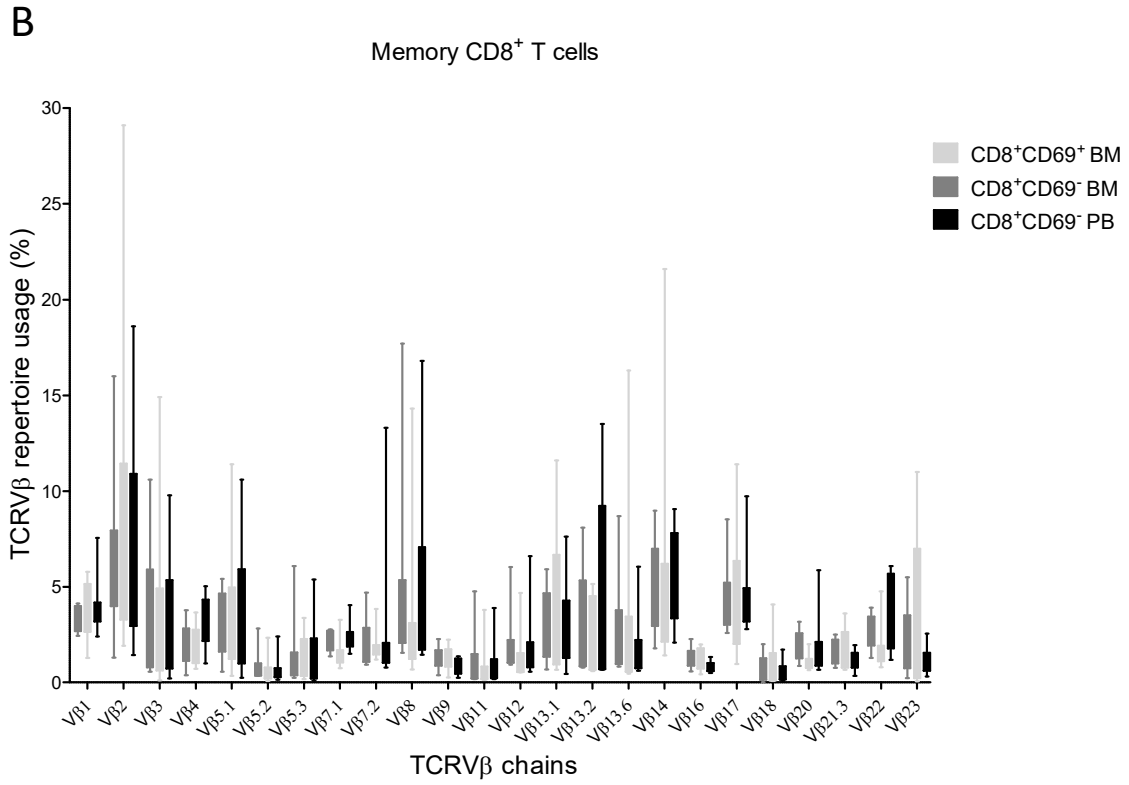


Figure 3-11: Comparison of the TCRβ repertoire in CD4⁺ (A) and CD8⁺ (B) memory T cell subsets from PB and BM. Mononuclear cells were isolated from paired blood and BM samples. Cells were directly stained with 24 TCRβ-specific monoclonal antibodies. Individual TCRβ expression was analyzed on gated CD69⁺ and CD69⁻ memory CD4⁺ T cells by flow cytometry. Average frequencies for each of the TCRβ families are shown in the box plots with Min to Max Whiskers (n=8). Statistical differences were calculated by 2-WAY ANOVA with Turkey correction ($p<0.0332$ (*), $p<0.0021$ (**), $p<0.0002$ (***), $p<0.00001$ (****)).

3.2.3 CDR3 TCRβ sequencing reveals different compartmentalization of memory T cells

Flow cytometric analysis of the TCR repertoire using antibodies specific for 24 Vβ families identified in humans provided us with an overall view of TCR usage. However, this did not account for the plethora of individual clones within each family. CDR3 TCRβ Next Generation Sequencing (NGS) allowed me to identify and quantify the distribution and expansion of individual T cell clones in PB and BM of an individual. The hypervariable complementary determining region 3 (CDR3) of the TCRβ chain is largely responsible for determining T cell specificity. The nucleotide sequence of CDR3 is unique for each T cell clone and thus can be used to track it and quantify its abundance in human PB and BM. I sorted CD4⁺ and CD8⁺ memory T cells (both CD69⁺ and CD69⁻) from paired PB and BM

samples and determined the different CDR3 sequences of the TCRVβ-chains in 4 donors. I chose RNA as starting material in order to analyze only functional molecules. Moreover, I introduced Unique Molecular Identifiers (UMIs) to all samples. UMI barcoding controls the numbers of molecules that have successfully entered cDNA synthesis and passed through amplification, which Ire then analyzed in the sequencing output. Such quantification is important to be able to perform normalized comparison of repertoires, as it excludes errors due to biased amplification during the PCR amplification steps[185]. To control for experimental variation in the library preparation and sequencing, samples from 1 donor were split following RNA extraction. Library preparation, NGS and analysis were performed in parallel to control for the robustness of the method. For the final analysis the data for this sample were pooled again.

First of all, I analyzed the clonal distribution of all replicates to determine the robustness of the method and the diversity of all studied samples. Technical replicates showed similar numbers of found sequences and clonotypes (Appendix table 6.2). I

I found 31-34% overlap between memory $CD4^+$ replicates, whereas the overlaps between memory $CD8^+$ technical replicates was higher than 83% in all cases (Figure 3.12). Even though the overlap between memory $CD4^+$ T cell replicates is low, the high overlap observed between the memory $CD8^+$ T cell samples indicates high reproducibility of the technical method used. When looking at the TCRβ diversity of analyzed samples, $CD4^+$ memory T cells showed a more diverse repertoire compared to the $CD8^+$ memory T cells (Appendix table 6.2). On average, the 10 most abundant clones found in all $CD4^+$ memory T cell subsets occupied less than 10% of the clonal space, whereas in $CD8^+$ memory T cells the clonal space occupied by the 10 most abundant clones was higher than 80%. These observations were also supported by the *Shannon eveness index (SEI)* of each sample, which is defined as a diversity index and quantifies how equal a population is numerically[186]. Memory $CD4^+$ T cells presented a *SEI* ranging from 0.96 to 0.98, meaning that all clonotypes found in the samples are almost equally distributed. On the other hand, the *SEI* for memory $CD8^+$ T cells was between 0.24 and 0.46, suggesting an unequal distribution of clonotypes found and less clonal diversity (Appendix table 6.2). Results obtained from the analysis of diversity in different cell subsets suggest that the low overlap found between memory $CD4^+$ T cells replicates may be due to technical limitations when working with highly diverse samples, as I do not see this effect among memory $CD8^+$ T cell replicates.

After that, I pooled the sequences from both technical replicates and analyzed the clonal space overlap between PB and BM memory T cell subsets.$CD4^+$ memory T cells showed low overlap between all 3 cell subsets. In all cases, more than 75% of the clonal space of each sample was exclusive for each cell subset. In the depicted representative donor, 20-25% of the clonal space was shared between $CD69^+$ and $CD69^-$ BM memory $CD4^+$ T cells; whereas the overlap between BM and PB $CD4^+$ memory T cells was lower than 10-15% in both cases (Figure 3.13A). Additional donors analyzed showed similar observations (Appendix figure 6.2). These results indicate that $CD69^+$ and $CD69^-$ BM $CD4^+$ memory T cells are more similar between them than compared to their PB counterparts, supporting the data obtained from the flow cytometric analysis. $CD8^+$ memory T cells showed a higher overlap between all memory T cell subsets (PB $CD69^-$, BM $CD69^-$ and BM $CD69^+$). I found 50 to 75% of overlap between all BM and PB memory $CD8^+$ T cell subsets with similar overlaps between all 3 memory T cell subsets (Figure 3.13). Additional donors analyzed showed similar observations (Appendix figure S6.2). Altogether, these data suggest that, at least in the $CD4^+$ population, PB and BM memory T cells form separated cell populations containing a different TCRβ repertoire, and that $CD69^+$ and $CD69^-$ cells from BM contain a more similar TCRβ repertoire between them, being closer between them compared to their PB counterparts.

Results obtained from the analysis of the total memory compartment indicate that different memory T cell populations are maintained separately in their respective compartments. However, the memory T cell pool is very extensive and diverse, so I performed a more accurate study by analyzing antigen-specific memory $CD4^+$ T cells from both compartments. To determine whether antigen specific memory $CD4^+$ T cells recognizing different epitopes are also preferentially maintained in different PB or BM, I analyzed the TCRβ repertoire of TT-specific $CD4^+$ memory T cells from both sites. Mononuclear cells Ire stimulated with TT (7 hours) and TT-reactive $CD4^+$ memory T cells Ire sorted according to CD154 and CD69 co-expression. Sorted TT-specific memory $CD4^+$ T cells were then sequenced by NGS and analyzed as performed before. In the depicted representative donor, I found 15% of overlap between PB and BM CDR3 sequences from TT-specific memory $CD4^+$ T cells (Figure 3.13). Similar results Ire observed in additional 2 donors analyzed (Appendix figure S6.2). These results suggest that there is little to no exchange between TT-reactive memory $CD4^+$ T cells from PB and BM compartments.

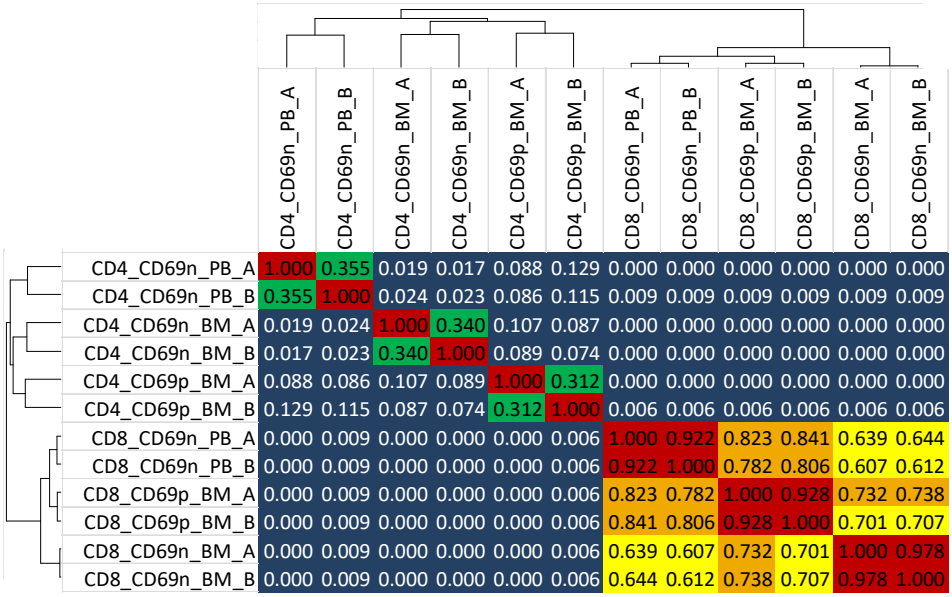


Figure 3-12: Analysis of TCR CDR3 clonotypes of ex vivo isolated memory CD4⁺ and CD8⁺ T cells. Memory T cell subsets from different samples were sorted according to the phenotype indicated and library preparation and data analysis was performed as detailed in material and methods. Overlaps between replicates and cell subsets were determined. Frequencies of overlaps between TCRβ CDR3 clonotypes from technical replicates and different memory T cells.

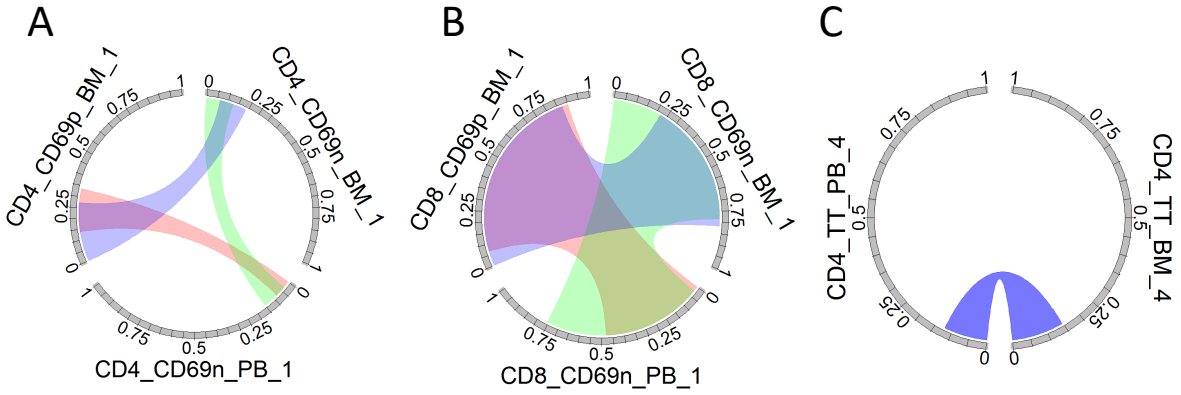


Figure 3-13. Clonal space shared between memory T cell subsets from PB and BM samples. TCR CDR3 sequencing was performed as described before. CDR3 sequences obtained from replicates were pooled and down-sampled. Frequencies of the clonal space shared between different memory T cell subsets are represented as circular graphs. (A) Frequencies of overlap between memory CD4⁺ T cell subsets from PB and BM sorted according to CD69 expression. (B) Frequencies of overlap between memory CD8⁺ T cell subsets from PB and BM sorted according to CD69 expression. (C) Frequencies of overlap between TT-specific memory CD4⁺ T cells from PB and BM.

3.3 Mobilization of human antigen-specific T_{RM} cells into blood after systemic immune re-challenge

In humans, it is still unclear whether and how T_{RM} cells can be mobilized to blood circulation after antigen re-challenge to mount a secondary immune response. To investigate which is the contribution of T_{RM} cells to systemic recall immune responses, I performed a longitudinal study of the memory $CD4^+$ T cell response to the live attenuated measles-mumps-rubella (MMR) viral vaccine in PB of healthy adult donors. Measles, mumps and rubella viruses, are a highly infectious RNA virus that infects humans through the respiratory tract, resulting in a systemic immune response in the host and long-life antiviral immunity[187,188]. 24 volunteers were screened for the presence of IgG-specific antibodies for measles, mumps and rubella and undetectable or low pre-existing numbers of antigen-specific memory $CD4^+$ T cells in blood circulation prior to vaccination. Volunteers selected had had an initial exposure to at least one of the vaccine antigens (via infection or vaccination) (Appendix table 6.3) over 30 years ago, reason why they maintained positive levels of IgG antibodies whereas the numbers of blood circulating MMR-specific memory $CD4^+$ T cells were very low[38,59] .

The experimental design used to probe the early memory response to MMR vaccination and characterize the response is depicted in Figure 3.14.

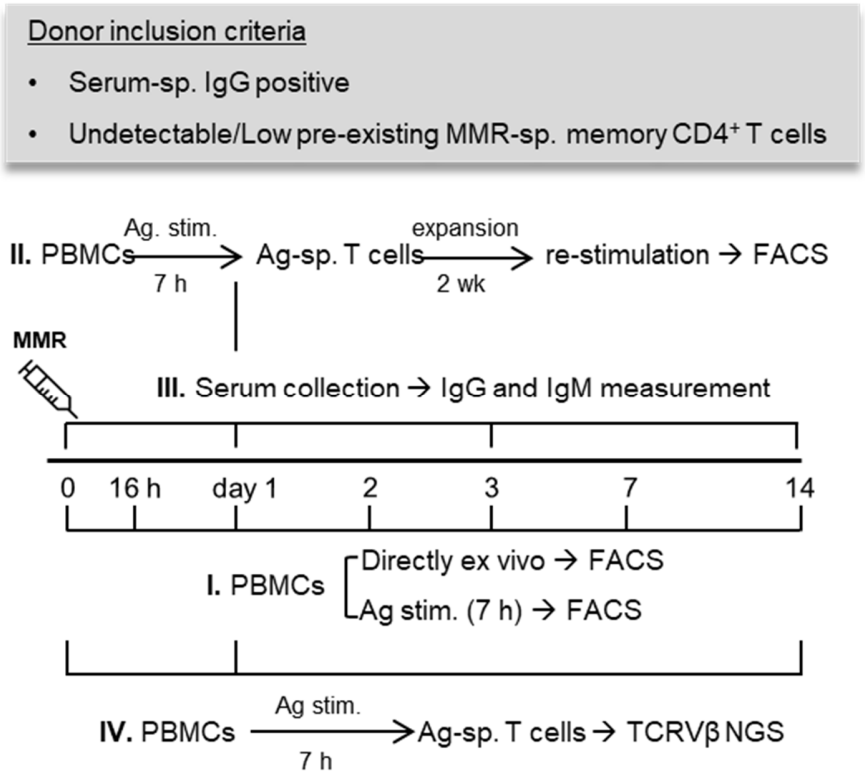


Figure 3-14: Experimental design for the study of the dynamics, functionality and specificities of the memory CD4⁺ T cell response after MMR vaccination. 24 healthy volunteers were screened to fulfill the donor inclusion criteria and suitable donors were re-vaccinated with and combined MMR vaccine. (I) Isolated PBMCs were directly analyzed or following in vitro stimulation with the vaccine-antigens. Afterwards, surface and intracellular flow cytometric analysis was performed. In all analyses, subtraction of anti-CD28 background was performed. (II) PBMCs isolated at day 1 after vaccination were stimulated for 7 hours in the presence of measles or TT antigens, and antigen-specific memory CD4⁺ T cells were isolated and expanded for 2 weeks. Expanded cells were re-stimulated for 6 hours with an array of antigens and FACS analysis was performed to identify reactive cells. (III) Serum samples were collected at the indicated time-points and antigen-specific IgM and IgG neutralizing antibodies were measured by ELISA. (IV) Measles-specific memory CD4⁺ T cells were isolated as described in II before and at days 1 and 14 after MMR vaccination to perform Next Generation Sequencing (NGS) of the TCR Vβ-chain.

3.3.1 Kinetics and magnitude of antigen-reactive memory CD4⁺ T cells after MMR vaccination

To probe the early memory response to MMR vaccination, I analyzed the kinetics and magnitude of MMR-reactive memory CD4⁺ T cells before, 16 hours, and days 1, 2, 3, 7

and 14 after MMR vaccination. MMR-reactive memory $CD4^+$ T cells were identified according the expression of CD154, and the fraction of cytokine-producing cells was determined by the co-expression of CD154 and one or more of the anti-viral effector cytokines IL-2, TNF-α and IFNγ (Gating strategy in Figure 2.5 materials and methods). By applying Boolean gating, single-, double-, and triple-cytokine producing cells were addressed.

With our protocol, I was able to detect in a reliable and reproducible way, as low as 0.01% of $CD154^+$ $Cytokine^+$ cells among memory $CD4^+$ T cells; and the threshold for differences was 0.005% between measurements (Appendix figure 6.3). In combination with the blood $CD4^+$ T cell counts (Appendix figure 6.4), the protocol allowed us to reliable quantify as low as 50 $CD154^+$ $Cytokine^+$ cells per 10^6 $CD4^+$ T cells.

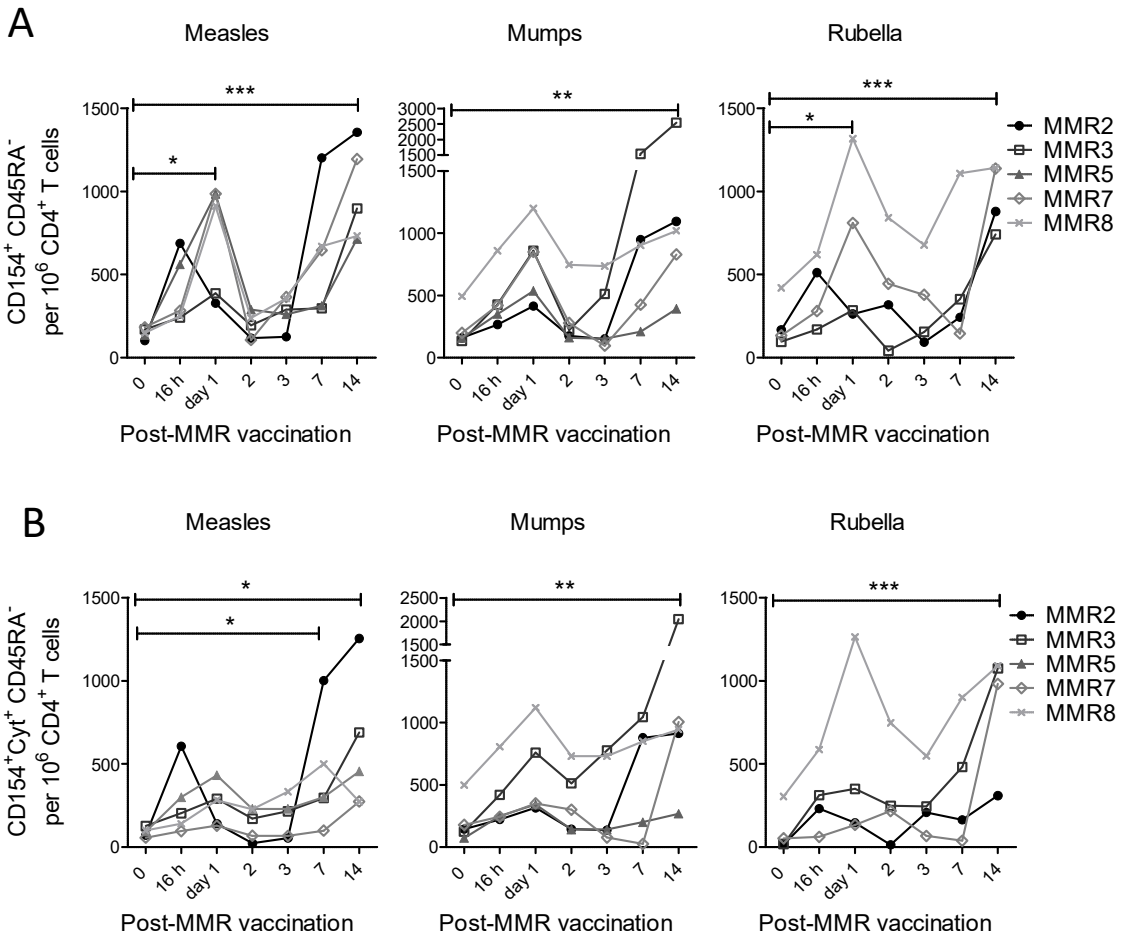
The dynamics of the MMR-specific memory $CD4^+$ responses over 14 days following MMR vaccination are shown for 5 representative donors (Figure 3.15A-C). Each vaccine-specificity is plotted separately. Prior to MMR vaccination, the numbers of pre-existing total measles-reactive $CD154^+$ memory cells were 100-200 per 10^6 $CD4^+$ T cells. For mumps and rubella, similar numbers were found in 4 out of 5 donors. Despite of the differential levels of pre-existing $CD4^+$ T cell memory in blood circulation, MMR vaccination induced similar but independent $CD4^+$ T cell responses to all antigens (Figure 3.15-3.16). For all specificities, a rapid increase in the numbers of antigen-reactive memory $CD4^+$ T cells was detected in blood as early as 16 hours, peaking at day 1 (from a MEAN ± SE of 164 ± 31.58 at day 0, to 349.1 ± 46 at 16 h and 624 ± 88 at day 1 after vaccination). At days 2-3, the numbers of antigen-reactive memory $CD4^+$ T cells declined to 225 ± 56 and 262 ± 49 respectively, further increasing to 696 ± 115 and 991 ± 137 at days 7 and 14 after MMR vaccination (Figure 3.15A).

To quantify the effector response, I analyzed the numbers of total $CD154^+cytokine^+$ memory cells per million $CD4^+$ T cells. The average numbers of $CD154^+cytokine^+$ memory $CD4^+$ T cells followed similar kinetics to the ones observed before, with a 2-fold increase at 16 hours, 3 fold increase at day 1 and 3- 5 fold increase at days 7 and 14, respectively, compared to the numbers of antigen-reactive memory T cells present before vaccination (Figure 3.15B).

Finally, to further quantify the effectiveness of the $CD4^+$ T cell response, I analyzed the dynamics of polyfunctional cells (expressing 2 or 3 cytokines) after MMR vaccination. The kinetics and magnitude of $CD154^+$ polyfunctional $cytokine^+$ memory $CD4^+$ T cells were

similar to the ones observed for total antigen-reactive CD154$^+$ memory CD4$^+$ T cells (Figure 3.15C).

Among all donors and specificities, I found a maximum of ~8 fold increase on antigen-reactive total-cytokine$^+$, and ~20 fold increase polyfunctional memory CD4$^+$ T cells at early time-points after vaccination (16 h and day 1). Altogether, these results show a rapid and transient increase of antigen-reactive memory CD4$^+$ T cells in blood circulation after systemic re-immunization.



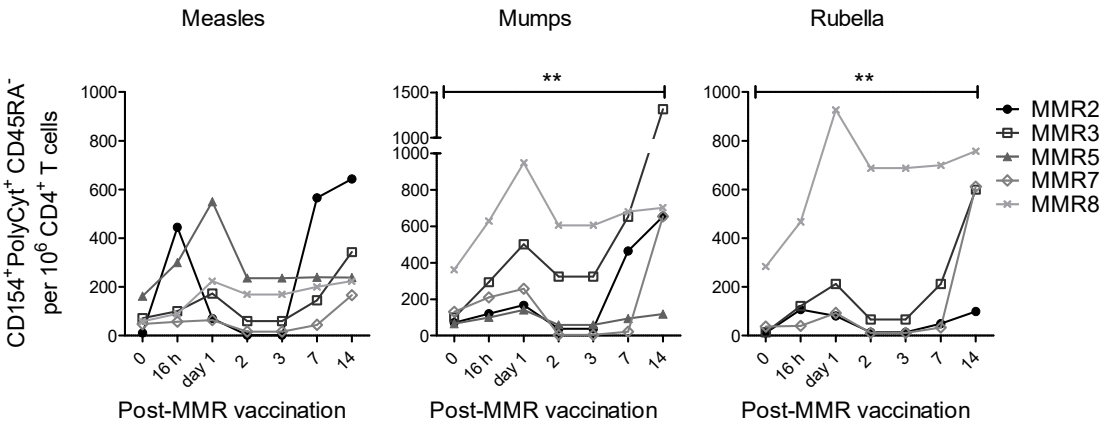


Figure 3-15: Cellular kinetics of antigen-reactive / memory CD4⁺ T cells after MMR immunization. After in vitro antigen stimulation and ICS of PBMCs isolated at different time-points, we analyzed the expression of CD154, IFNγ, TNFα and IL-2 and followed the dynamics of the numbers of CD154⁺ (B), CD154⁺ Cyt⁺ (C) and CD154⁺ Poly-Cyt⁺ (D) per million CD4⁺ T cells. 5 representative donors are shown for each of the specificities. Statistical differences were calculated by 2-WAY ANOVA with Turkey´s multiple comparisons test ($p<0.0332$ (*), $p<0.0021$ (**), $p<0.0002$ (***), $p<0.00001$ (****)).

Early appearing antigen-reactive CD4⁺ T cells were mostly polyfunctional, producing two or three cytokines at the same time. For all specificities, on average 68% of the total CD154⁺cytokine⁺ memory CD4⁺ T cells expressed more than one cytokine. Polyfunctionality has been associated with an increase in the cytokine content per cell, which is related with a higher protective capacity [189–191]. I compared the geometric mean (GEOM MEAN) of IFNγ on antigen-reactive CD154⁺cytokine⁺ memory CD4⁺ T cells that produced three, two, versus one cytokines, named 3, 2, versus 1 effector functions. Despite the presence of polyfunctional cells at all time-points analyzed, the most significant differences in cytokine content were detected only at 16 hours and day 1 after MMR vaccination. At 16 hours and day 1 after vaccination, double and triple producers contained in average 2 times and 3 times more IFNγ, respectively, compared to the single-producing cells. Contents of IL-2 and TNFα in double and triple producers were also increased in a similar way as described for IFNγ (data not shown). Both, the major proportion of polyfunctional antigen-reactive CD4⁺ T cells and the higher cytokine production from double and triple-cytokine producers suggest that early mobilized cells provide an immune response of enhanced quality.

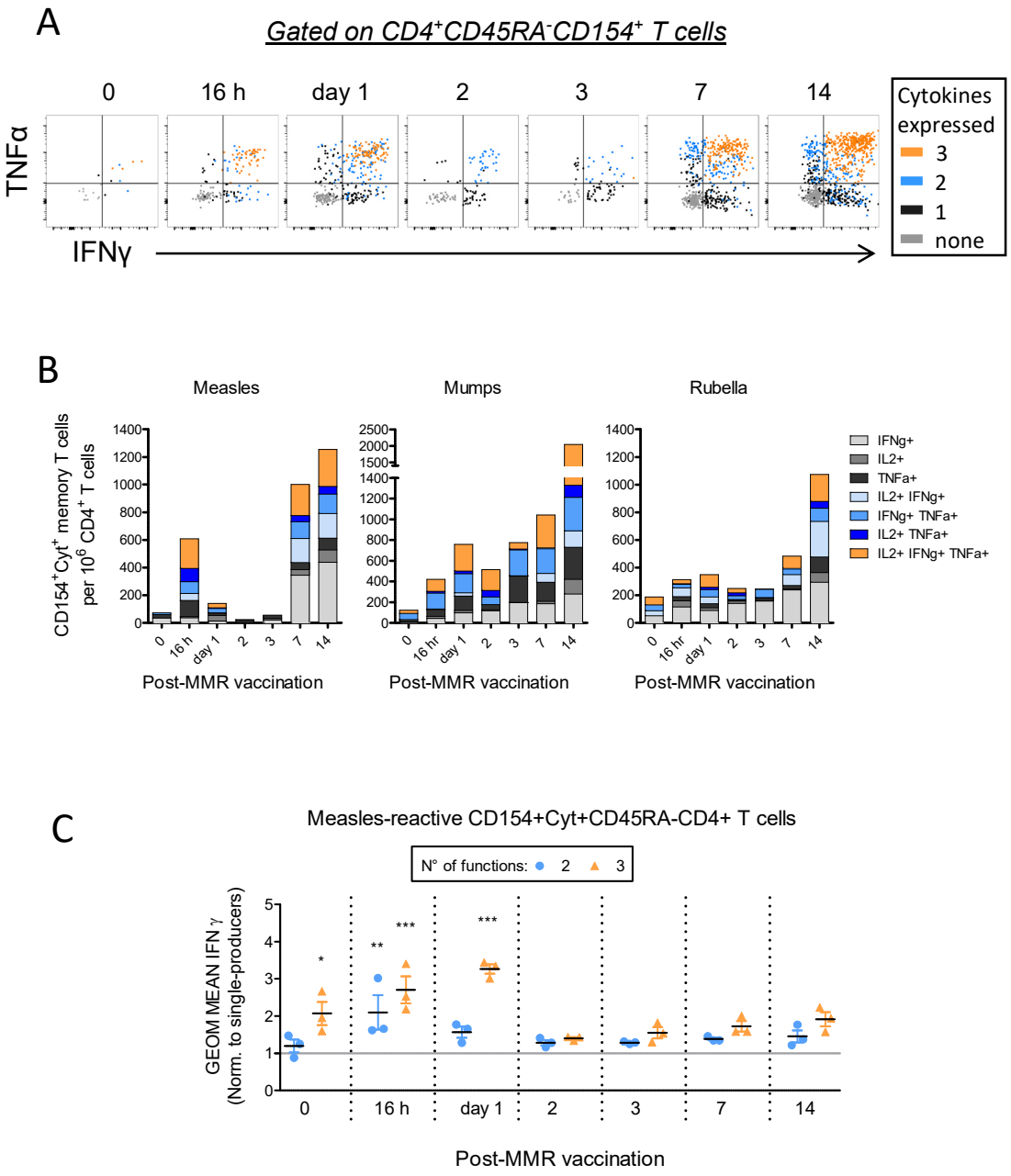


Figure 3-16: Cellular kinetics and functional capacities of antigen-reactive memory CD4⁺ T cells after MMR immunization. Using in vitro antigen-stimulation with the indicated antigens and ICS I followed the frequencies of CD154⁺Cyt⁺ memory CD4⁺ T cells present in blood at indicated time-points. (A) Representative dot-plots with magnitude and number of functions of antigen-reactive memory CD4⁺ T cells appearing after MMR immunization. (B) Dynamics of the secondary response to measles, mumps and rubella, showing the number of single, double and triple cytokine producers in a representative donor. (C) Geometric mean fluorescence intensity (GMFI) of IFNγ in double and triple producers normalized to the GMFI detected in single cytokine producers. Statistical differences were calculated by 1-WAY ANOVA with Turkey correction.

A part from analyzing the immune responses in antigen-experienced volunteers, I had the opportunity to analyze the primary response to MMR vaccination in a naïve donor. The analysis of the immune response of a measles-naïve donor (with non-detectable levels of both measles-specific IgM and IgG antibodies as well as non-existing measles-reactive memory $CD4^+$ T cells in blood circulation) showed a response different from the one observed in antigen-experienced donors, with the appearance of $CD154^+Cytokine^+$ (1500 cells per million $CD4^+$ T cells) and $CD154^+Ki\text{-}67^+$ (2100 cells per million $CD4^+$ T cells) measles-specific $CD4^+$ T cells at day 14 after MMR vaccination, together with an increase of both IgG and IgM specific antibodies (Figure 3.17A). These results are in line with the results obtained from the kinetics of primary T cell response observed in yellow fever vaccine studies[192,193]. Interestingly, the response measured for the other two antigens present in the vaccine showed the above-mentioned dynamics of a secondary immune response, and I detected only and increase on the levels of IgG specific antibodies at day 14 after vaccination, reflecting a secondary immune response (data not shown).

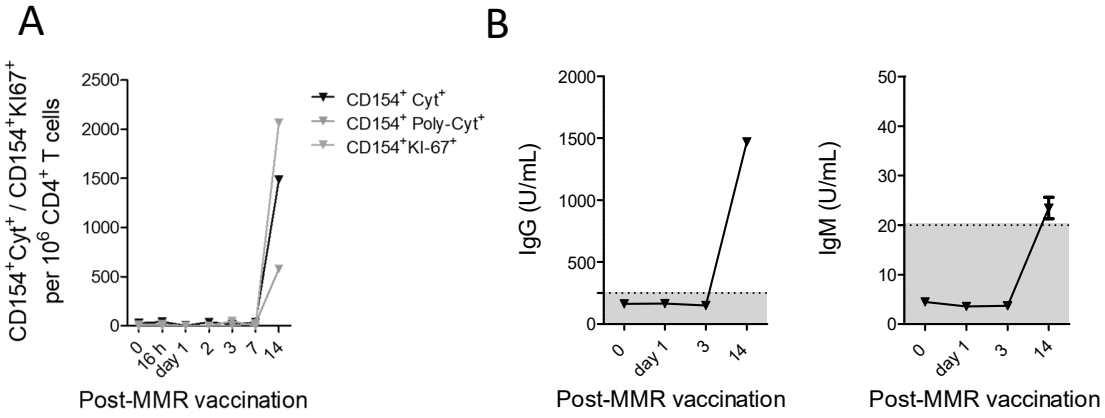
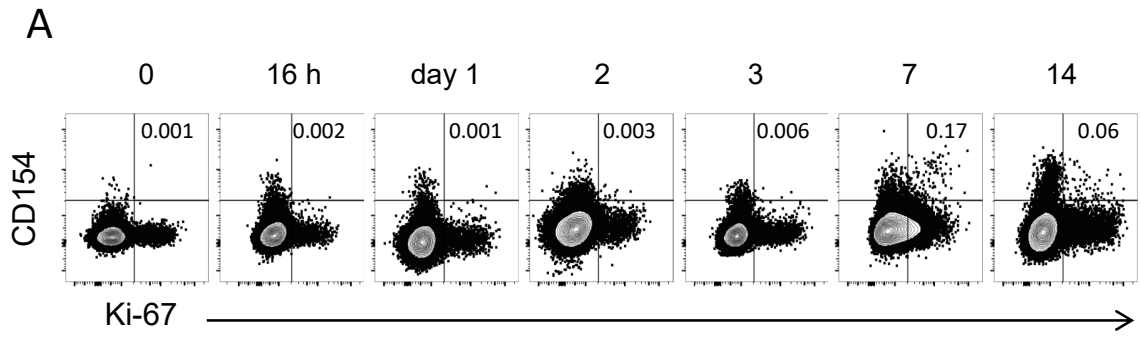


Figure 3-17: Kinetics of a naïve response to MMR vaccination. Cellular and humoral kinetiks were determined as described before. (A) Total and polyfunctional $CD154^+Cyt^+CD45RA^-$as well as $CD154^+Ki\text{-}67^+CD45RA^-CD4^+$ T cells dynamics in response to MMR vaccination. (B) Levels of both IgG and IgM measles neutralizing antibodies were measured at the indicated timepoints by ELISA.

3.3.2 MMR vaccine-induced early antigen-reactive memory CD4⁺ T cells contain mobilized T_{RM} cells

Compared with pre-existing circulating T cell memory, the increase on antigen-reactive memory T cells detected at 16 hours/day 1 after MMR vaccination suggest an influx of memory T cells either from proliferated pre-existing memory T cells or from a hidden distinct population of T_{RM} cells that are mobilized into the blood circulation. In order to test these two scenarios, I measured expression of the proliferation marker Ki-67[194] in MMR-reactive CD154⁺ memory CD4⁺ T cells before and after MMR vaccination. Cells in G_0 of their cell cycle do not express Ki-67, whereas cycling or recently divided T cells up-regulate Ki-67 expression [194,195].

In our study, Ki-67 was not expressed by any of the measles-, mumps- and rubella-reactive memory CD4⁺ T cells before (0) and at the early time-points (16 hours, and days 1, 2 and 3) after vaccination. Antigen-reactive memory CD4⁺ T cells were only detectable from 7 days (236 ± 48) and 14 days (153 ± 33) after MMR vaccination (Figure 3.18A-B). These results demonstrate that the rapid increase of circulating antigen-reactive memory CD4⁺ T cells after MMR vaccination is a result of the mobilization into blood circulation of memory T cells resident in other compartments, such as the BM, instead of expansion of pre-existing circulating cells.



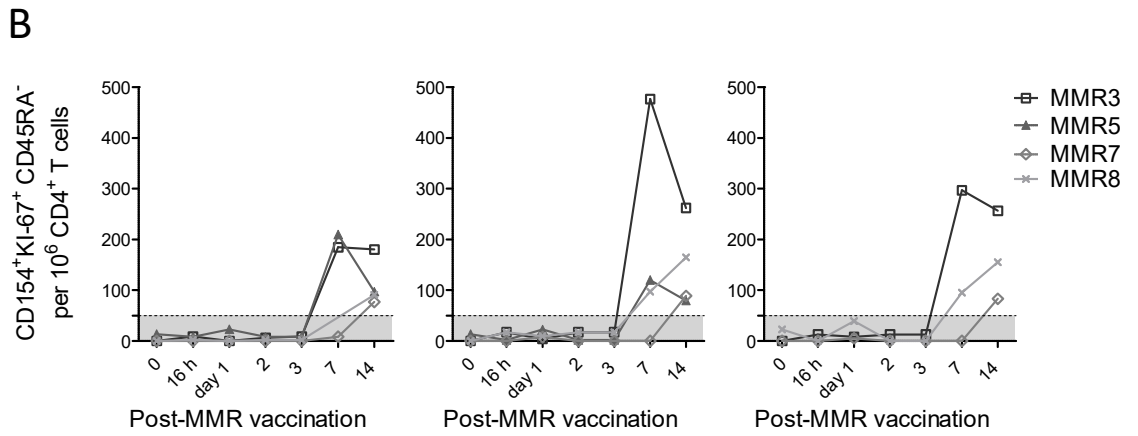


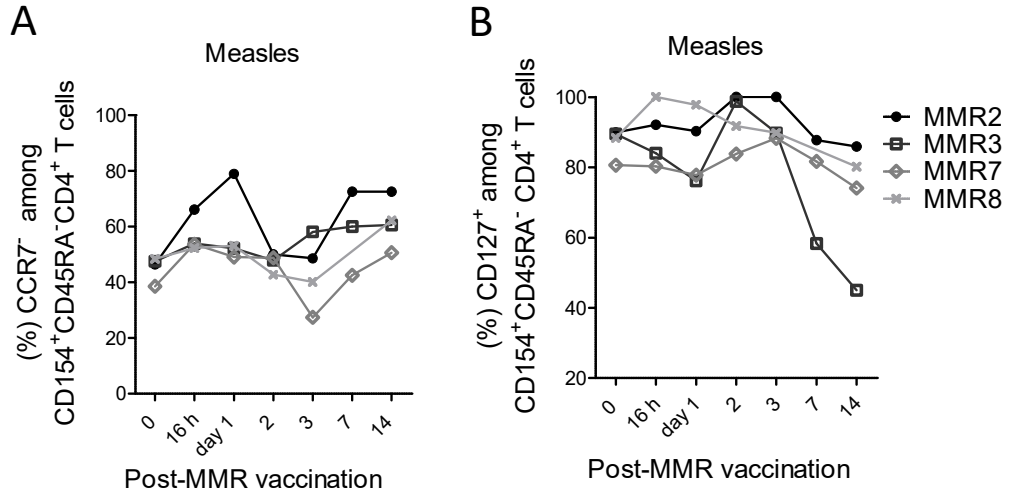
Figure 3-18: Kinetics of appearance of antigen-reactive proliferating cells (CD154$^+$ Ki-67$^+$) after MMR vaccination. Using ex vivo stimulation with the indicated antigens and ICS I followed the numbers of CD154$^+$Ki-67$^+$ memory CD4$^+$ T cells present in blood at indicated time-points. (A) Representative dot-plots with frequencies of proliferating antigen-reactive memory CD4$^+$ T cells appearing after MMR immunization.(B) Numbers of antigen-reactive CD45RA$^-$CD154$^+$Ki-67$^+$ T cells per million CD4$^+$ T cells of 4 representative donors for each of the antigen specificities.

3.3.3 Early mobilized MMR-reactive memory CD4$^+$ T cells show mainly a T_{RM}/T_{EM}, memory precursor-like phenotype

To further characterize the memory CD4$^+$ response, I determined whether MMR mobilized MMR-reactive memory CD4$^+$ T cells phenotypically resembled functional T_{RM} cells. For that, I analyzed the expression of CCR7 on antigen-reactive memory CD4$^+$ T cells before, and at different time-points after immunization (Figure 3.19A). It is know that, T_{RM} resemble T_{EM} cells, being able to provide with immediate enhanced effector functions and do not express the chemokine receptor CCR7 [196]. T_{CM} have the capacity to circulate through secondary lymphoid organs, whereas T_{EM} and T_{RM} preferentially localize in peripheral tissues[61,197]. The frequencies of CCR7$^-$ cells among measles-reactive memory CD4$^+$ T cells increased from 45 ± 2.25% before vaccination to 56.5 ± 3.22% and 58.3 ± 6.9% at 16 hours and day 1 after immunization, respectively. The levels dropped to levels seen before vaccination at days 2 and 3, rising again to 56 ± 6.5% and 61.5 ± 4.5% at days 7 and 14 after vaccination, respectively (Figure 3.20B). These results indicate that early mobilized antigen-reactive memory CD4$^+$ T cells mostly resemble T_{RM}/T_{EM} cells, with no expression of CCR7 and high expression of effector cytokines, such as IFNγ and TNFα (Figure 3.16B).

High expression of the IL-7Rα and the anti-apoptotic molecule BCL-2 are correlated with high survival potential, and tend to decrease in recently generated effector CD4⁺ T cells [198]. Our results showed that 80-90% of the measles-reactive memory CD4⁺ T cells expressed this marker on the surface at the early time-points after vaccination, with only a slight decrease in expression one week after vaccination (56-85% CD127⁺ among measles-reactive T cells) which was more prominent 14 days after vaccination (35-85% CD127⁺ among measles-reactive T cells) (Figure 3.19B). These results suggest that early mobilized antigen-reactive memory CD4⁺ T cells maintain a high survival potential, whereas the later increase of antigen-reactive memory CD4⁺ T cells may contain a pool of newly generated effector cells with low CD127 expression.

Finally, to gain a better understanding of the functions of the mobilized CD4⁺ T cells, I determined the expression of various cell surface molecules on the antigen-reactive CD4⁺ T cells. Phenotypic analysis performed by surface staining with CD137, PD-1 and CXCR5 revealed that there was no mobilization of antigen-specific T regulatory or T follicular helper cells into the blood as a consequence of MMR vaccination (Appendix figure 6.5). Moreover, the analysis of expression of CLA, (a typical marker for skin homing) showed a constant frequency of ~20% among measles-reactive memory CD4⁺ T cells at all time-points analyzed (except at day 14, where the frequencies of CLA⁺ cells increased to 32%) (Figure 3.19C). Although these results should be confirmed in further experiments, it indicates that early mobilized antigen-specific memory CD4⁺ T cells were not derived from a skin resident memory T cell pool.



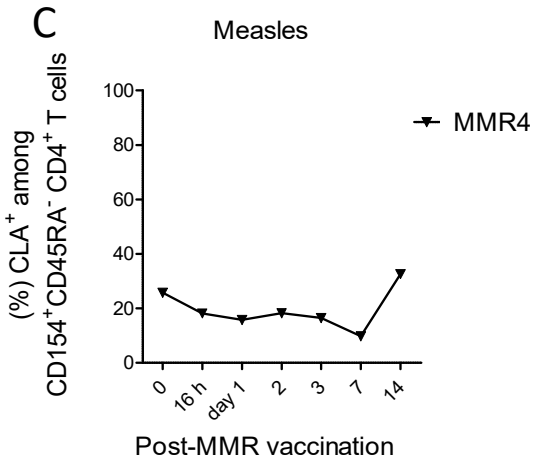


Figure 3-19: Phenotypic analysis of antigen-reactive CD4$^+$CD45RA$^-$CD154$^+$ T cells. Using ex vivo stimulation with the indicated antigens I performed a phenotypic analysis of CD154$^+$ memory CD4$^+$ T cells present in blood. Frequencies of CCR7$^-$ (A), CD127$^+$ (B) and CLA$^+$ (C) cells among antigen-reactive CD154$^+$ memory CD4$^+$ T cells.

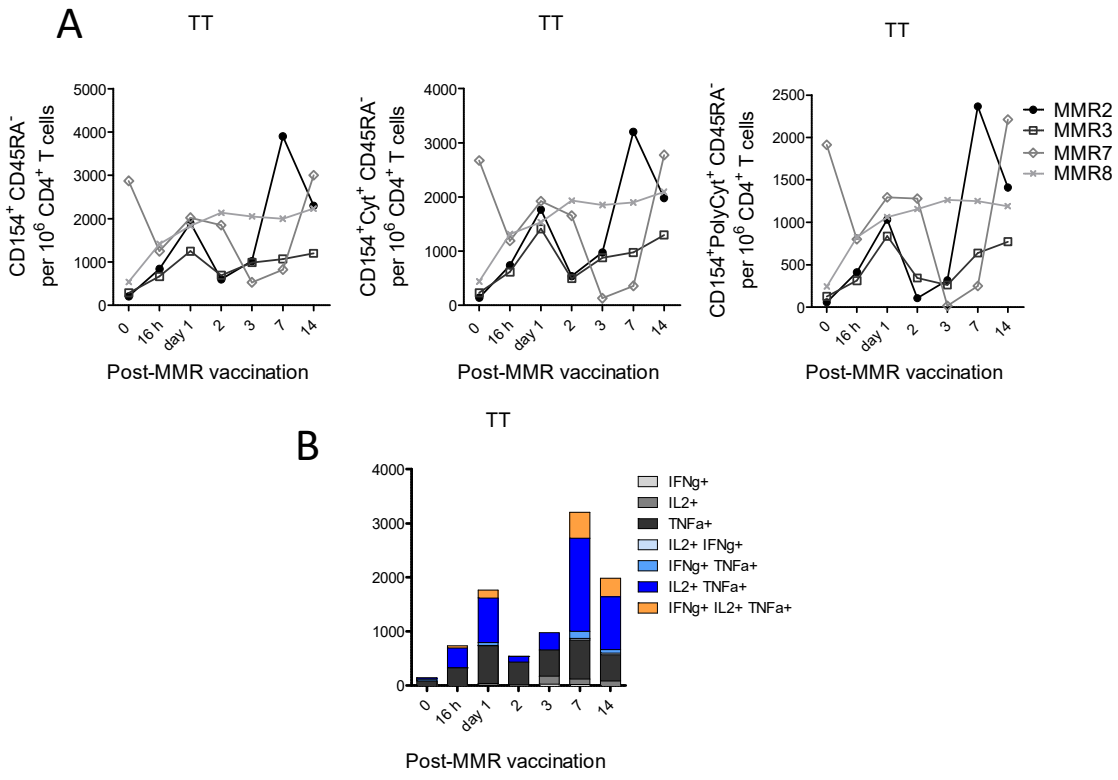
3.3.4 MMR vaccination induces bystander mobilization of TT-specific memory T cells with similar dynamics to the vaccine-specific response

Di Genova and colleagues have previously shown that TT boost vaccination of healthy volunteers results in an expansion of both, TT-specific T cells but also of T cells with other specificities [199]. To understand whether MMR vaccine also trigger bystander memory T cell responses to unrelated antigens, I analysed the time course of TT-reactive memory T cell responses after MMR vaccination. Similar to measles, mumps, and rubella, at 16 hours and day 1 after vaccination, numbers of total TT-reactive CD154$^+$, CD154$^+$cytokine$^+$, and CD154$^+$ polyfunctional cytokine$^+$ memory CD4$^+$ T cells were all readily and transiently increased by 2.5-19-flod, dropping to baseline by day 2/3 and increasing again by day 7/14 in three out of four donors (Fig. 3.20A). Bystander mobilized memory CD4$^+$ T cells were also polyfunctional, mainly secreting IL-2 and TNFα, but low levels of IFNγ (Figure 3.20B). These results suggest that, after MMR vaccination, bystander mobilization of unrelated antigen-specificities occur in parallel with the MMR-specific memory CD4$^+$ T cell response (Figure 3.20A).

Previous studies have shown that vaccine-reactive and bystander mobilized memory CD4$^+$ T cells differ in their proliferative status, as well as their expression of markers such as

CCR7 and CD127 1-2 weeks after boost vaccination [200]. In order to better characterize the bystander mobilized memory CD4⁺ T cells, and distinguish them functionally and phenotypically from MMR-specific memory CD4⁺ T cells, I analyzed the expression of Ki-67, CCR7 and CD127 in TT-reactive memory CD4⁺ T cells. Our results showed low expression of the proliferation marker Ki-67 TT-reactive memory CD4⁺ T cells at any time-point of the study, i.e. below 2% Ki-67⁺ among $CD4^+CD54RA^-CD154^+$ TT-reactive T cells (Figure 3.20C). Frequencies of $CCR7^-$ cells were maintained (~50%) among $CD4^+CD54RA^-CD154^+$ TT-reactive memory CD4⁺ T cells (Figure 3.20D). Finally, TT-reactive memory CD4⁺ T cells showed high expression (80-90%) of the IL-7Rα (CD127) at all time-point analyzed (Figure 3.20E).

Altogether, these results show that early bystander mobilized TT-reactive memory CD4⁺ T cells show a similar phenotype compared to vaccine-specific memory CD4⁺ T cells, with no expression of Ki-67 and high expression of CD127, but with a more central memory-like phenotype. However, 1-2 weeks after vaccination, bystander mobilized cells show differences in their phenotype compared to the MMR-reactive memory CD4⁺ T cells, with no signs of increased proliferation and no down-regulation of CD127 expression.



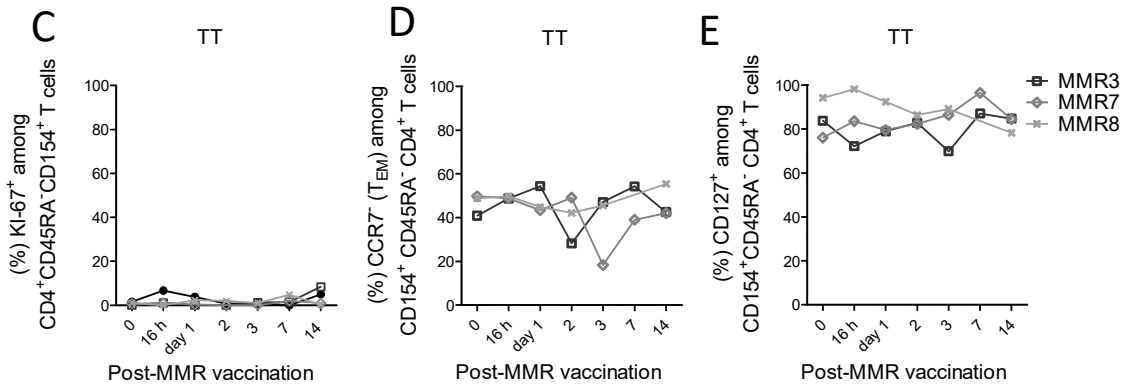


Figure 3-20: Dynamics and phenotypical characteristics of bystander mobilized TT-specific memory CD4+ T cells. (A) After in vitro TT stimulation and ICS of PBMCs isolated at different time-points, I analyzed the expression of CD154, IFNγ, TNFα and IL-2 and followed the dynamics of the numbers of CD154+, CD154+ Cyt+ and CD154+ Poly-Cyt+ per million CD4+ T cells. 4 representative donors are shown. (B) Representative dynamics of the specific secondary response to TT, analyzing the number of single, double and triple cytokine producers in a representative donor. (C) Frequencies of Ki-67$^+$ cells among TT-specific memory CD4$^+$ T cells (D) Frequencies of CCR7$^-$ cells among TT-specific memory CD4$^+$ T cells (E) Frequencies of CD127$^+$ cells among TT-specific memory CD4$^+$ T cells before and at indicated time-points after MMR vaccination.

3.3.5 The early MMR-reactive memory CD4$^+$ T cells and the antibody response are highly specific for the vaccine antigens

To verify the specificity of MMR vaccine responses and exclude the possibility that the observed mobilization and response against unrelated antigen (TT) were due to cross-reactivity, I evaluated the memory T cell responses as well as antibody responses to vaccine antigens or unrelated antigens. I isolated measles- and TT-reactive CD154$^+$CD69$^+$ memory CD4$^+$ T cells from day 1 after MMR vaccination. Day 1 antigen-reactive memory CD4$^+$ T cells were expanded for 14 days[201], and re-stimulated with autologous APCs and the indicated antigens. As shown in Figure 3.21A-B, measles-reactive, expanded CD4$^+$ T cells specifically reacted to the inducing antigen, whereas reactivity against other antigens, present in the vaccine (mumps and rubella) or not (TT and CMV) was at background level (Figure 3.21A). Cytokine-producing T cells were mostly polyfunctional, expressing TNFα and IFNγ, or TNFα, IFNγ and IL-2 (Figure 3.21A-B). After background subtraction, 8-20% of the expanded T cells expressed CD154 and at least one of the three analyzed cytokines. When re-stimulating with other antigens present in the MMR vaccine (mumps and rubella), only 0,5-1,5% of the expanded T cells reacted. No reactivity was observed

when the expanded T cells were re-stimulated with TT or CMV (Figure 3.21C). On the other hand, 6% of expanded TT-reactive CD4⁺ memory T cells were reactive to TT re-stimulation, showing cross-reactivity to measles in one of the analyzed donors and to CMV in the other two ones (Figure 3.21C). These data indicate little to no cross-reactivity and high specificity of the early mobilized vaccine-reactive memory CD4⁺ T cells, whereas the bystander mobilized TT-reactive memory CD4⁺ T cells seem to include multiple specificities.

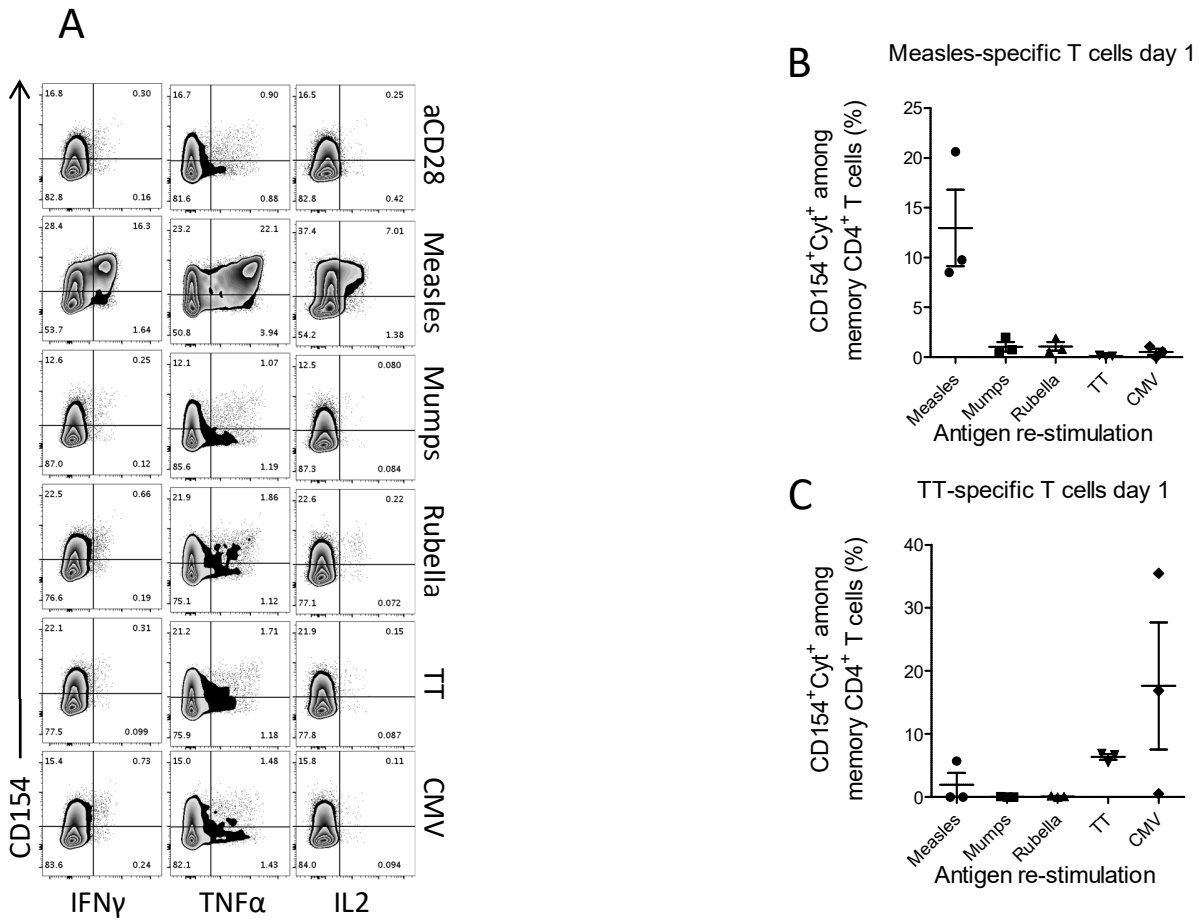


Figure 3-21: Specificities of early appearing antigen-reactive memory T cells. Measles-reactive memory CD4⁺ T cells isolated at d1 after vaccination were expanded with proleukin (IL-2) for 14 days and then re-stimulated with indicated antigens and aCD28 for 7 h and stained ICS with CD154, IL2, IFNγ and TNFα . (A) Representative dotplots of cytokine expression after stimulation with different antigens. (B) Percentages of CD154⁺Cyt⁺ among memory CD4⁺ T cells after stimulation of measles-reactive CD4⁺ T cells (n=3). (C) Percentages of CD154⁺Cyt⁺ among memory CD4⁺ T cells after stimulation of d1 TT-reactive CD4⁺ T cells expanded for 14 days (n=3).

To correlate the MMR-induced memory T cell responses with the corresponding antibody responses, as we as assess the specificity of the antibody response, I analyzed the magnitude of measles, mumps, rubella and TT-specific antibodies before, and at days 1, 3 and 14 after MMR vaccination. In all pre-immunized subjects, who were serum-positive for measles-, mumps- and rubella-specific IgG antibodies before vaccination, I detected a 2- to 4-fold increase of IgG-titers at day 14 after re-immunization. IgM levels were below level of detection at all time-points (Figures 3.22A-B). In contrast to MMR, no increase in TT-specific IgG antibodies was observed in any of the analyzed donors, which remained remarkably stable (Figure 3.22B). These results demonstrate that MMR vaccination induces a highly specific antibody response.

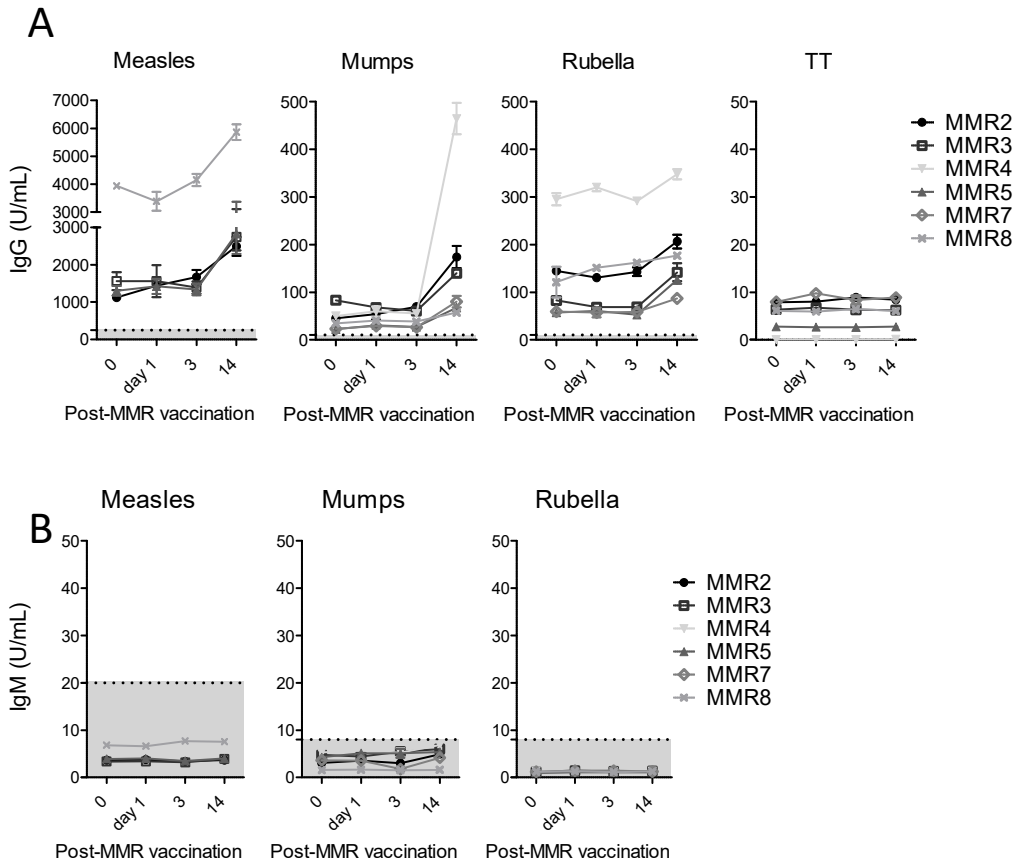


Figure 3-22: Kinetics of neutralizing antibody response to MMR vaccination. Serum from immunized donors was obtained at indicated time-points and the levels of IgG and IgM antibodies specific for measles, mumps, rubella and tetanus toxoid (TT) were analyzed by ELISA. (A) IgG levels detected at different time-points for measles, mumps, rubella and TT. (B) Levels of IgM antibodies specific for measles, mumps and rubella specificities.

3.3.6 The early mobilized MMR-reactive memory CD4⁺ T cells suppose an influx of new TCR Vβ diversity, which contributes to the immune response

Previous studies performed in humans demonstrated there is a preferential enrichment of antigen-reactive memory CD4⁺ T cells for childhood antigens in the BM [59]. Moreover, the TCRβ sequencing shown in section 3.2.3 indicate that PB and BM memory CD4⁺ T cells represent different populations according to TCRβ-chain repertoires (section 3.2.3).

In order to get an insight about the origin of the mobilized antigen-reactive memory CD4⁺ T cells, as well as their contribution to the secondary immune response, I analyzed the CDR3 TCRβ repertoire of measles-reactive memory CD4⁺ T cells. The CDR3 sequences of 2500 sorted CD154⁺CD69⁺ measles-reactive memory CD4⁺ T cells were determined in two healthy individuals immunized with the MMR vaccine before, and at days 1 and 14 after vaccination. Both donors showed low numbers of measles-specific memory CD4⁺ T cells before vaccination, which were increased at both days 1 and day 14 after re-immunization (similar to previous results when analyzing further donors). I found an increase in CDR3 TCRβ diversity at day 1 after immunization compared to day 0 in both studied volunteers (139 ± 25 / 322 ± 9 to 346 ± 37 / 613 ± 21 of clonotypes found at day 0 and 1 respectively), which was then maintained in MMR10 and decreased to basal levels in MMR11 at day 14 (Figure 3.24A). To estimate the total number of unique TCR Vβ CDR3 sequences present in the sampled blood, rarefaction curves were calculated by Pawel Durek, and a computational approach developed by Fischer et al. [203] and subsequentially extended by Efron and Thisted [204] was used. This method has already been used to estimate the total CDR3 TCRβ diversity of human blood samples [205,206], and has been demonstrated to provide a reasonable estimate of the total diversity of CDR3 TCRβ sequences in the repertoire [205]. Based in the assumption that T cells circulate freely in the blood, this method predicts the number of unseen sequences that would be detected if the experiment was repeated an infinite number of times. Efron and Thisted indexes of diversity varied in both donors from 329 ± 64 / 971 ± 5 to 924 ± 109 / 1795 ± 17 and 1223 ± 127 / 926 ± 32 at days 0, 1 and 14 respectively (Appendix table 6.4). Moreover, compared with day 0, day 1 measles-reactive memory CD4⁺ T exhibited higher sampled diversity at orders from 1 to 10, as indicated by Hill numbers, which quantify the diversity in units of equivalent numbers of equally abundant species (Figure 3.24B). These results

suggest that early mobilization of antigen-reactive memory CD4⁺ T cells induced by MMR vaccination results from an influx of new CDR3 clonotypes.

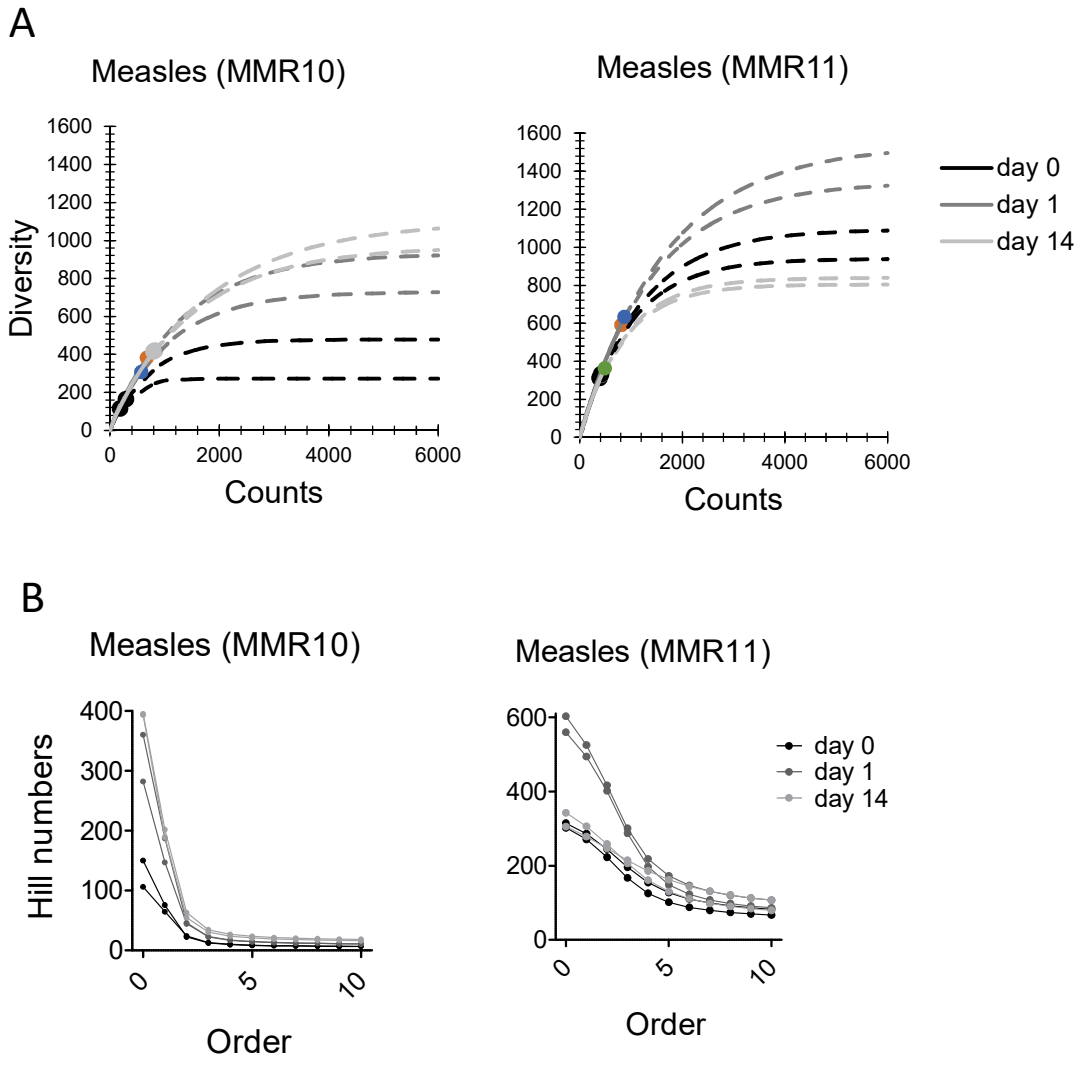


Figure 3-23. Influx of new CDR3 TCR clonotypes from MMR-mobilized measles-specific CD4⁺ T_{RM} cells persist and continue by day 14 post-vaccination. 2500 measles-specific memory CD4⁺ T cells were isolated after in vitro stimulation before, and at days 1 and 14 after MMR vaccination. TCR Vβ CDR3 nucleotide sequences obtained by NGS from 2 technical replicates were analyzed. (A) Rarefaction curves were calculated. Individual points indicate the number of clonotypes found in each sequenced sample, and dashed lines predicts the number of unseen sequences that would be detected if the experiment was repeated an infinite number of times. (B) Diversity of clonotypes at different time-points is plotted at different orders. At order 0, the Hill numbers are the number of clonotypes found. At higher orders (>1) the Hill numbers are more influenced by the most abundant clones

I further assessed the appearance of new CDR3 clonotypes at day 1, which could still be found at day 14 post-vaccination. For that, I identified abundant clones that were undetectable before vaccination, but that were constantly present in both replicates of days 1 and 14 after MMR vaccination. I found 6 and 5 clonotypes in donnor MMR10 and MMR11 respectively, which appeared on day 1 and were still present at day 14. In MMR10, the clonal space occupied by newly appearing clonotypes increased from ~2% at day 1 to ~7 % at day 14 after vaccination. 2 out of 6 clonotypes were expanded 4 fold at day 14 compared to day 1, whereas the others were maintained at similar frequencies. MMR11 showed similar frequencies of all clonotypes at both time-points, occupying ~2% of the total clonal space (Figure 3.25). In order to determine the contribution of pre-existing clones to the generation of the secondary immune response, I also analyzed the numbers and frequencies of the clonotypes present before, and at days 1 and 14 after MMR vaccination. In this case, I did not observe any expansion of the abundant clonotypes already present at day 0 in any of the analyzed donors (Figure 3.25).These results show that, systemic re-challenge with the MMR vaccine induces an influx of new CDR3 TCRβ clonotypes, which are maintained and presumably play a role in the generation of the secondary immune response. Newly appearing clonotypes may appear in blood circulation as a result of the mobilization of tissue resident memory T cells from other tissues, such us the BM.

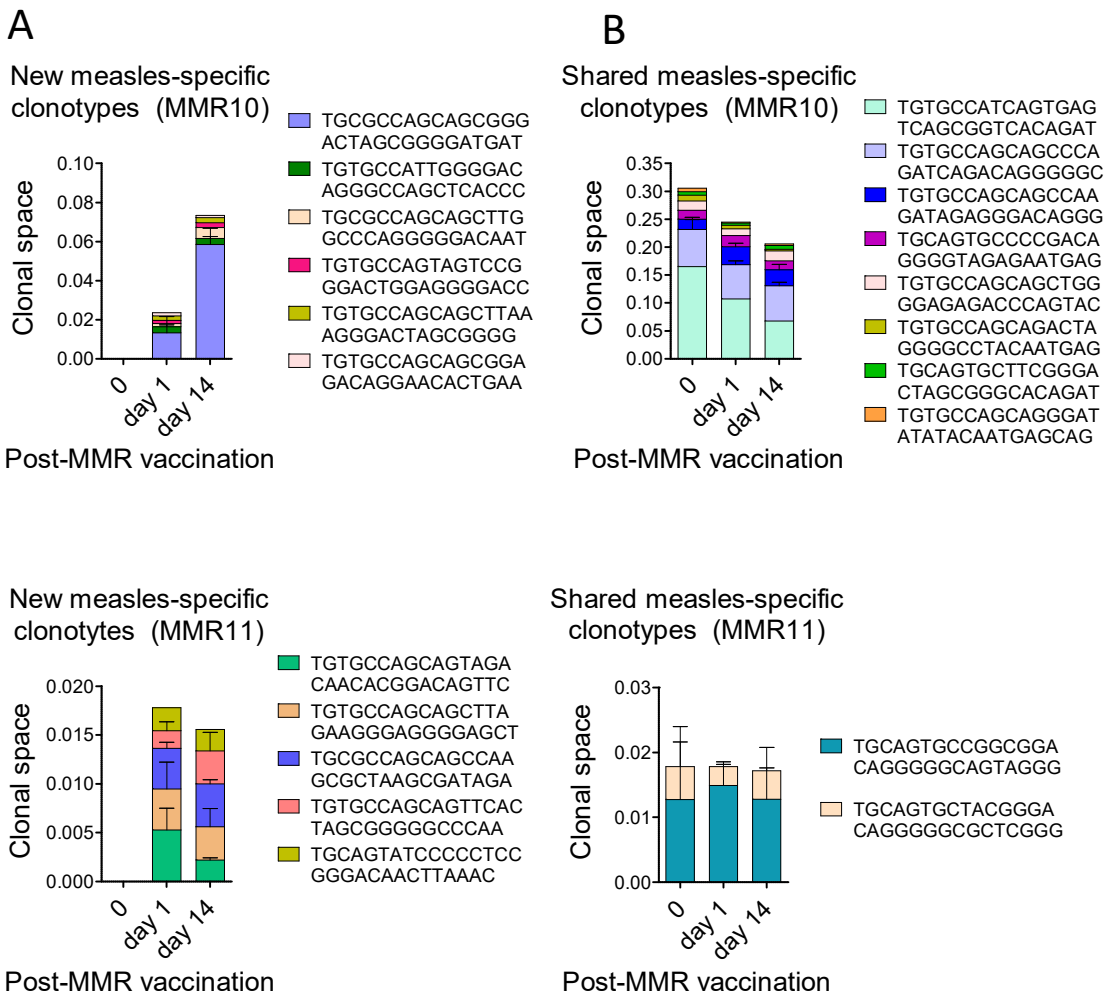


Figure 3-24. Analysis of the contribution of newly generated clones to the secondary immune response. Abundant clones present in both of the replicates were analyzed. (A) Identification and frequences of clonotypes not present before vaccination and constantly present at days 1 and 14 after vaccination. Data of two independent donors is shown. (B) Identification and frequences of clonotypes constantly present before and at days 1 and 14 after vaccination. Data of two independent donors is shown.

4 Discussion

Immunological memory provides us with efficient and directed protection against secondary infections, having the capacity to clear re-occurring pathogens. Memory T cells play an essential role in sustaining long-term immunological memory. These cells have been identified in PB circulation but also resident in different tissues such as the skin, gut and the bone marrow [59,72–76,90]. Previous work of our group showed that the human BM is home to high numbers of professional memory T cells, and that it is especially enriched for memory $CD4^+$ T cells specific for systemic childhood antigens like measles, mumps and rubella[59]. However how memory T cells are maintained for such long times in the BM, what is the relationship the relationship between circulating and resident memory T cells, and whether and how tissue resident memory T cells are they capable to confer us with systemic immune protection after immune re-challenges remained not fully understood.

The experimental work presented here aimed to better understand the different lifestyle and compartmentalization of PB circulating versus BM resident memory T cells, as well as the contribution of T_{RM} cells (such as the ones resident in the BM) to secondary systemic immune reactions in humans.

I found differences in the survival mechanisms of PB versus BM memory T cells, as well as some of the key factors involved in the maintenance of these cells. Moreover, the analysis of the TCRβ repertoire of PB and BM memory T cells by flow cytometric analysis and NGS demonstrated their different compartmentalization in the respective tissue. Finally, the analysis of the dynamics of MMR-specific memory $CD4^+$ T cells into blood after systemic immune re-challenge in the long-term (i.e. 30 years) showed a rapid mobilization into blood circulation of antigen-reactive memory $CD4^+$ T cells from other tissues (such as the BM), which then contribute to the secondary immune response.

4.1 PB and BM memory T cells present different survival patterns, and are maintained via cytokines (IL-7 and IL-15) and direct contact with stromal cells

4.1.1 Different lifestyles of circulating versus BM memory T cells

PB circulating and BM resident memory T cells are subjected to different environments. PB memory T cells circulate through the blood and secondary lymphoid organs or tissues. Some studies suggested that most mature lymphocytes recirculate continuously from blood to tissue and again blood almost 1-2 times per day [207]. On the other hand, our group has provided evidence that BM resident memory T cells are maintained in a quiescent state in specific survival niches[59], where stromal and other accessory cells provide them with signals to survive [90,208,209]. Several studies have suggested that CD69 regulates local T cell retention in the BM by a variety of mechanisms [125,128,210]. Considering PB and BM memory T cells as different cell populations submitted to very distinct environments, they may present different survival capabilities without or in response to diverse survival factors.

The survival kinetics of ex vivo PB $CD69^-$, BM $CD69^{-/+}$ memory $CD4^+$ and $CD8^+$ T cells showed that BM memory T cells (both $CD4^+$ and $CD8^+$) survived to a lesser extent and had reduced half-life compared to their PB counterparts, being the $CD69^+$ BM cell subset the one with shortest half-life. PB memory T cells must have higher capacities to survive in the absence of any survival stimulus while they are circulating through the bloodstream, where the concentrations of cytokines such as IL-7 or IL-15 are low[211]; whereas BM memory T cells may be continuously receiving these signals from their environment, being therefore more dependent on them. The fact that $CD69^-$ BM memory T cells are in the middle of PB and $CD69^+$ BM memory T cells regarding survival capacity could suggest they may have the capability to enter the circulation in some cases, instead of being always in the BM survival niches.

In addition, I tested the effect of the oxygen concentrations in the survival of PB and BM memory T cells. It has been estimated that in humans, arterial blood has a pO_2 = 13.2, whereas this is reduced in venous blood (pO_2 = 5.3) and in BM (pO_2 = 6.4±0.6) [159]. Therefore, the “normal” oxygen levels in which experiments are typically performed (pO_2=21) may not be adequate for any of the populations analyzed, especially for BM memory T cells. I compared the survival kinetics and half-lives of PB $CD69^-$ and BM

$CD69^{-/+}$ when cells were cultured in 21% and 4% O_2. Our results showed that both $CD69^-$ and $CD69^+$ BM memory T cells had an increased half-life when cultured at lower oxygen levels, whereas PB $CD69^-$ memory T cells maintained similar survival capacities in either O_2 level.

Effector memory $CD4^+$ T cells recirculate between normoxic blood and hypoxic tissues to screen for cognate antigens. Circulating memory T cells, thus, face and must adapt to different environmental conditions while circulating through the blood, or passing through secondary lymphoid organs and tissues (where the levels of oxygen are reduced[159]). This fact could explain that there was no obvious differences in survival when culturing the cells under 21% or 4% O_2. BM memory T cells are maintained in a quiescent state in specific survival niches [90], where they adapt to the low levels of oxygen present into the tissue. This fact explains why BM memory T cells had increased survival rates when submitted to low oxygen concentrations (4%) compared to 21% oxygen. Differential survival of PB and BM memory T cells at different oxygen concentrations indicate that indeed, they are different populations maintained in their respective compartment, where they adapt to the local environment to survive.

A recent publication investigating the survival and metabolism of skin T_{RM} $CD8^+$ memory T cells demonstrated increased mitochondrial oxidative metabolism of these cells in the presence of exogenous fatty acids (FA), compared to T_{CM} memory T cells. Moreover, the persistence of $CD8^+$ T_{RM} cells in the skin was strongly diminished by inhibition of mitochondrial FAβ-oxidation. When comparing transcriptomes of T_{RM} and T_{CM} cells, an up-regulation of the genes encoding lipid chaperone proteins that bind to hydrophobic ligands coordinating lipid uptake and intracellular trafficking[212] (FABP4 and FABP5) was detected. The lack of FABP4 and FABP5 in T_{RM} cells resulted in lower lipid uptake and survival of tissue resident memory T cells[213]. Similarly, when looking at transcriptomes of human $CD4^+$ memory T cells from paired PB and BM samples[59], I could detect an increase on FABP5 expression in BM $CD69^+$ memory T cells compared to the PB counterparts (≈ 3-fold increase), suggesting their metabolism resembles to the one found in T_{RM} cells, and is different from their PB counterparts. A more detailed understanding of the metabolic programs intrinsic to BM resident and circulating memory T cells should be investigated in order to see how these programs could be manipulated to increase or decrease T cell memory longevity and function.

4.1.2 Maintenance of resting BM memory T cells by IL-7 and IL-15

Some reports showed an increased proliferation of BM memory $CD8^+$ T cells by bromodeoxiuridine (BrdU) labelling in murine BM[214,215], and interpreted these data as evidence for maintenance of memory $CD8^+$ T cells by homeostatic proliferation. However, studies from our group support the idea that long-lived BM memory T cells are maintained in a quiescent state, with no signs of activation, migration or proliferation[59,90]. Moreover, studies performed in mice eliminating proliferating cells by cyclophosphamide treatment have further demonstrated that BM memory $CD8^+$ T cells are resting in terms of proliferation[216].Furthermore, our group has recently demonstrated that BrdU feeding itself induces proliferation of memory $CD8^+$ T cells [135]. From these studies, we could conclude that BM memory T cells are maintained in a quiescent state, with no signs of proliferation.

γ-chain cytokines, such as IL-7 and IL-15 have been shown to be involved in the maintenance of circulating memory $CD8^+$ T cells [145,146] and memory $CD4^+$ T cells, with some discrepancies in the latest[144,147,148]. However, it is still not completely clear what role these two cytokines play in the maintenance of T_{RM} cells. For instance, T_{RM} isolated from the brain are incapable of surviving outside their tissue niche [81] and do not appear to use IL-7 or IL-15 for survival [96],. On the other hand, recent studies using skin T_{RM} cells demonstrated that hair-follicle-derived IL-7 and IL-15 are required for $CD4^+$ and $CD8^+$ T_{RM} cells to persist in the epidermis [217]. It remains unknown whether there are common survival factors that maintain T_{RM} in all tissues, or whether each tissue provides its own unique signal. In the context of this thesis, I aimed to confirm whether IL-7 and IL-15 play an important role in the maintenance of BM memory T cells. The addition of each cytokine to BM T cell cultures resulted in an improvement in their survival, demonstrating their involvement in the long-term maintenance of BM memory T cells.

Regarding the question of how memory T cell is maintained in the BM, some studies have shown that IL-7 and IL-15 can induce $CD8^+$ T cell proliferation [149,214,215]. However, the amounts of cytokines used in these studies are usually high (above 10 ng/mL), and probably not reflecting the physiological conditions found in the BM. In our experiments, I could confirm that high concentrations of IL-7 and IL-15 (10 ng/mL) induced proliferation of memory T cells *in vitro*. However, concentrations until 1 ng/mL of IL-7 and IL-15 were able to maintain memory T cell survival without signs of proliferation (no blasts, increase in cell numbers and Ki-67 expression). It would be important to determine the local concentrations of both cytokines in the BM stromal survival niches in order to better

understand the conditions to which memory T cells are subjected locally and how these influence their maintenance.

Apart from IL-7 and IL-15; there is less information about the existence of other factors that could be involved in the long-term maintenance of BM memory T cells. For this reason, I aimed to better characterize the factors involved in the maintenance of BM memory T cells. IL-6 is also present in high amounts in the BM and it is an important survival factor for memory plasma cells, another long-lived memory cell type resident in the BM[218,219]. Other cytokines like IL-33, TNFα, TGFβ and type I interferons have also been implicated in the generation and persistence of T_{RM} cells [92] and seem to have a role in the maintenance of skin and gut resident memory T cells [87,220]. However, in our *in vitro* experiments, only IL-7 and IL-15 showed an effect in the maintenance of BM memory T cells.

The fact that IL-6 does not play a role in the survival of memory T cells like it does for plasma cells suggests that different memory cell types are maintained in specialized stromal survival niches. This could be reflected in the heterogeneity of stromal cells in the BM. Long-lived memory plasma cells translocate into the BM and dock onto stromal cells that express CXCL-chemokine ligand-12 (CXCL12 or SDF1) and vascular cell-adhesion molecule 1 (VCAM1 or CD106) [136,208], memory $CD4^+$ and $CD8^+$ T cells were shown to be in direct contact with $VCAM^+$ IL-7 producing stromal cells [90,135]. Moreover, in humans, $CD8^+$ memory T cells were also found in clusters with IL-15 producing cells [78]. These findings indicate that different memory T cell types are maintained in different specialized niches, which will provide the essential signals for each cell type to be kept alive.

On the other hand, IL-33 and TGFβ were shown to be important during the generation and persistence of the T_{RM} phenotype[221], but did not have any effect on the survival of BM memory T cells. These data suggest that the role of IL-33 and TGFβ is limited to the first phase of establishment of the T_{RM} cells, and does not extend to the long-term maintenance of those cells. However, we cannot exclude their importance on maintaining T_{RM} cells from other tissues, as I only tested them in BM memory T cells. Further experiments using T_{RM} from other tissues may be needed to better understand whether cells from different tissues are maintained by similar or different factors.

In conclusion, our results showed that both IL-7 and IL-15, but none of the other cytokines tested supported the survival of both $CD4^+$ and $CD8^+$ BM memory T cells. A more detailed histological analysis of human BM sections would help to determine the heterogeneity of

the BM stromal niches and to identify additional factors involved in the long-term maintenance of memory T cells.

4.1.3 In addition to IL-7and IL-15, stromal cell contact also play an important role in maintaining quiescent memory T cells

I further tested the effect of IL-7 and IL-15 in PB and BM patients in both, $CD4^+$ and $CD8^+$ memory T cells in order to investigate whether both cytokines would have the same or different effects in all memory T cell subsets. In all BM T cells, both cytokines were able to significantly improve the survival of memory T cells. The survival of PB memory T cells was also increased with both cytokines to a similar extent than to BM memory T cells, suggesting that IL-7 and IL-15 are also important in the maintenance of circulating memory T cells. Interestingly, the analysis of 27 multiplexed serum cytokine concentrations in healthy subjects showed the existence of low concentrations of IL-7 (≈ 50 pg/mL) and non-detectable levels of IL-15 [211] in human serum samples, suggesting that circulating memory T cells may be receiving a boost of survival factors while going through secondary lymphoid organs or tissues in order to be kept alive.

IL-7 showed a major role in sustaining cell survival in $CD4^+$ compared to $CD8^+$ memory T cells; whereas IL-15 showed to be more important for the maintenance of memory $CD8^+$ T cells. IL-7 receptor (CD127) expression has been reported to be high and similar in both $CD4^+$ and $CD8^+$ memory T cells[152]. On the other hand, the receptor for IL-15 (CD122) is highly expressed on memory $CD8^+$ T cells compared to memory $CD4^+$ T cells [137], which could be related with the major influence of this cytokine on memory $CD8^+$ T cell but not so much in memory $CD4^+$ T cell survival [144,150,151]. Until now, it is not known whether $CD4^+$ and $CD8^+$ memory T cells are maintained in the same or different survival niches. However, from our data it seems that both cell populations have slightly different requirements for their maintenance, so the fact that they are maintained in different and separated BM survival niches could be possible. Again, further histological analysis of human BM tissue would be important to decipher the heterogeneity of BM survival niches and the contribution of each of them to the maintenance of different cell types.

Of note, both cytokines could not maintain 100% of memory T cell survival, suggesting that other factors provided within the stromal cell niche may have an additional role in supporting memory T cell long-term maintenance. Apart from soluble factors, cell-cell

interactions could also be important for the maintenance of memory T cells. BM resident memory T cells express both, VLA-2 and VLA-4, which bind to collagens I, II and XI [153,154] and VCAM-1[155,156] respectively. Interaction with VCAM-1 is important for the maintenance of hematopoietic stem cells [157] and plasma cells [158] in the BM. Moreover, memory $CD4^+$ T cells have been found in direct contact with $VCAM^+$ IL-7 producing stromal cells [90] by histological analyses of mouse BM sections. In order to test the role of T cell-stromal cell contact in memory T cell survival, I performed *in vitro* co-cultures of PB and BM memory T cells with the human stromal cell line HS5. Publicly available transcriptomes of the HS5 human stromal cell line (http://www.ncbi.nlm.niih.gov/geo) showed no expression of IL-7 and low expression of IL-15, but high expression of VCAM-1 and collagens. Luminex assays performed with the supernatants of the stromal cell line confirmed the absence of both cytokines, and flow cytometric analysis of VCAM-1 corroborated its high expression on the cell surface of HS5 cells. Co-culture of human memory T cells with HS5 supported to a similar extent their survival compared to the addition of IL-7 or IL-15. Interestingly, PB memory T cell survival was also improved when cultured with the stromal cell line, suggesting PB memory T cells are also capable to integrate pro-survival signals from cell-cell interactions when circulating through the secondary lymphoid organs or tissues.

In order to assure that stromal cells were not sustaining cell viability by the secretion of IL-7 or IL-15, I measured the expression of CD127 in all memory T cell subsets cultured in different conditions. After contact with IL-7, CD127 (the α-chain of the IL-7 receptor) is internalized and down-regulated as feedback control. It had been previously shown, that the incubation of human PBMCs with IL-7 decreased cell-surface expression of CD127 on $CD4^+$ [183] and memory $CD8^+$ T cells within 24-48 hours of culture [184]. Our results showed a total down-regulation of CD127 when memory T cells were cultured with IL-7 with also a slight down-regulation in the presence of IL-15. No decrease in the expression of CD127 was detected in memory T cells co-cultured with the stromal cell line, strongly suggesting that the BM HS5 stromal cell line is not supporting memory T cell survival via the expression of IL-7 or IL-15. In addition, transwell experiments performed by Jessica Dysarz (Bachelor student at the DRFZ) and cultures of memory T cells with BM stromal supernatants performed by Kathrin Stilz (Master student at the DRFZ) demonstrated that direct cell-cell interaction between memory T cells and HS5 cells was essential to maintain cell survival. Further experiments will be needed to elucidate which surface molecules of stromal cells are important for maintaining the survival of memory T cells. Blocking experiments with antibodies as well as genetic manipulation of the stromal cells to inhibit

the expression of candidate molecules should be performed in order to assure their implication in BM memory T cell survival.

Both cytokines and direct contact with stromal cells could not maintain 100% of memory T cell survival. In BM survival niches, all pro-survival signals may be integrated in order to maintain a constant population of memory T cells in a resting state. To test if cytokine and stromal signals could be integrated to maintain ≈ 100% of cells alive, I performed an experiment where BM memory T cells were co-cultured with HS5 cells and the cytokines. Our results showed that under these conditions, BM memory T cells were induced to proliferate (observed by cell blasting and increase on cell numbers at day 5), taking out the memory T cells from their resting state. The human HS5 BM stromal cell line shows high expression of the IL-7 receptor in its surface, so it is possible that the interaction of the cytokines with the stromal cells could cause changes on them, therefore inducing memory T cell proliferation. Until now, our *in vitro* system does not allow us to fully mimic the physiological conditions found in the BM niches, where memory T cells can be maintained for long-time periods in a resting state[59]. In order to obtain a more accurate system to mimic the *in vivo* BM environment, further experiments should be performed using autologous primary stromal cells. Since now, I was able to expand BM stromal cells *in vitro* for months and use them for some preliminary survival experiments, but there is a time-delay until the stromal cells are expanded and ready to be used in experimental procedures, challenging the performance of such experiments using autologous stromal and memory T cells.

In conclusion, our results show that IL-7, IL-15 and direct contact with stromal cells play a role on maintaining the survival of both, PB and BM memory T cells. The integration of both pro-survival mechanisms as well as additional ones triggered by factors yet unknown must be needed for the fully maintenance of resting long-term memory. The investigation of the closest neighbors in situ of the different populations of memory T cells in the specific BM stromal niches is needed to fully understand all the players involved in the maintenance of BM memory T cells.

4.1.4 Molecular mechanisms involved in the survival of memory T cells

So far, evidence suggests that multiple molecular signals derived from different cellular sources are needed to maintain memory T cell survival in the BM niche. However, it is not known how these signals are integrated to ensure memory T cell survival. IL-7 and IL-15 activate the JAK-STAT pathway which induces expression of anti-apoptotic BCL-2 family members, including BCL-2 and MCL-1[180,222].

Direct contact with stromal cells through a combination of adhesion molecules have also shown to be important for the persistence of some memory cell types in the BM. For instance, direct cell-cell interaction via VLA-4/VCAM-1 has been shown to be required for the survival of plasma cells in BM niches [223]. Signaling through VLA-4 results in the activation of the PI3K/Akt pathway, which could also be implicated on the maintenance of memory T cells.

To better understand which molecular signals are implicated in the maintenance of memory T cells, I analyzed the expression of anti-apoptotic (BCL-2 and MCL-1) and pro-apoptotic (BIM and NOXA) molecules in *in vitro* cultured BM and PB memory T cells. Our results show a significant increase on BCL-2 expression in all memory T cell subsets cultured with IL-7 and IL-15, resulting in a pro-survival balance on the BCL-2/BIM ratios. Moreover, the fact IL-7 induced BCL-2 expression to a greater extent in $CD4^+$ than in $CD8^+$ memory T cells, whereas IL-15 induced BCL-2 more strongly in $CD8^+$ memory T cells compared to their $CD4^+$ counterpart. These results are in concordance with the results obtained in the survival assays, and suggest that IL-7 has a major role in $CD4^+$ memory T cells survival whereas IL-15 is more important for the maintenance of $CD8^+$ memory T cells. Both cytokines acted similarly in all BM and PB T cell subsets, indicating that both BM resident and circulating memory T cells can equally respond to these signals with up-regulation of anti-apoptotic molecules. IL-7 and IL-15 induced only a non-significant increase in MCL-1 expression and had no influence on BIM and NOXA expression, indicating that they act primarily through the up-regulation of BCL-2.

Direct contact with stromal cells significantly increased the MCL-1/NOXA ratios by down-regulating NOXA expression in all PB and BM memory T cell subsets. NOXA has been related to the control of memory $CD4^+$ T cell homeostasis [176], and its expression is directly regulated by the p53 tumor suppressor protein. p53 is negatively regulated by Akt, which enhances degradation of p53 in a MDM2-dependent manner, thereby inhibiting cell apoptosis[224,225]. Therefore, cell-cell contact of memory T cells with stromal cells via

integrins may lead to Akt activation and p53 suppression, resulting in the final NOXA down-regulation and survival. The fact that both BM and PB memory T cells down-regulate NOXA upon stromal cell contact suggests that both PB circulating and BM resident memory T cells have the capacity to interact with stromal cells and respond with enhanced survival. BM memory T cells could be constantly receiving these pro-survival signals, as they are in direct contact with VCAM-1^{+}IL7^{+} stromal cells[77].

In conclusion, our results demonstrate that cytokines and direct cell-cell interaction with stromal cells promote cell survival via different but complementing mechanisms. Activation of γ-chain cytokine receptors signals through the JAK/STAT pathway, resulting in BCL-2 up-regulation, and thereby increasing the BCL-2/BIM ratio. On the other hand, direct contact with stromal cells could activate the PI3K/AKT pathway, leading to NOXA down-regulation, and a positive MCL-1/NOXA balance. The integration of both pro-survival signals thus results in the proper equilibrium of pro- and anti-apoptotic molecules, promoting memory T cell survival. Further experiments investigating the signaling pathways upstream of these molecules are needed to fully understand the molecular mechanisms required for the survival of memory T cells. Ablation of the different pro-survival pathways with chemical inhibitors would provide with information about which are the most important survival pathways in memory T cells. Moreover, the identification of additional pro-survival molecules as well as the molecular pathways activated by these ones is of great importance to fully decipher the survival mechanisms of BM memory T cells. The integrated information of signals required as well as molecular mechanisms implicated in the survival of memory T cells will be very useful in order to see how these survival programs could be manipulated to increase or decrease T cell memory longevity and function.

4.2 Compartmentalization of PB circulating versus BM resident memory T cells

4.2.1 BM CD69$^+$ and BM CD69$^-$ CD4$^+$ memory T cells maintain antigen specificities against long-term antigens

Previous studies of our group analyzing antigen-specific memory T cells in human PB and BM paired samples demonstrated an enrichment of memory CD4$^+$ T cells in BM compared to PB specific for systemic childhood antigens like measles, mumps or rubella, but also for other antigens like TT (recurring antigen) or CMV-pp65 (chronic antigen)[59]. In this study, I aimed to know whether these antigen specificities were maintained or not by both CD69$^+$ and CD69$^-$ memory CD4$^+$ T cells in the BM. Several studies have shown that T_{RM} cells are heterogeneous regarding their CD69 expression [59,71,226]. Studies focused on tissue resident CD8$^+$ T cells using parabiotic mice demonstrated that CD8$^+$ CD69$^-$ T cells were constantly recirculating, whereas tissue resident CD8$^+$ CD69$^+$ cells were non-recirculating[69,79]. In contrast, Steinert and colleagues have provided evidence that both CD69$^+$ and CD69$^-$ memory T cells are tissue resident [71]. These observations raised the question whether enrichment of memory T cells reactive to particular antigens in the BM are the result of specific accumulation within the CD69$^+$ cell subset or whether they are equally distributed between both cell CD69$^+$ and CD69$^-$ memory T cells. The distribution of measles and TT-specific CD4$^+$ memory T cells between the CD69$^+$ and CD69$^-$ CD4$^+$ memory T cell subsets was investigated in order to test it for both, a long-passed (measles) and a recurring (TT) antigen. Measles-specific memory T cells were equally distributed among the CD69$^+$ and CD69$^-$ cells. TT-specific memory T cells were also present in both cell subsets, suggesting a similar array of antigen specificities in both CD69$^+$ and CD69$^-$ memory CD4$^+$ T cells.

These results show that both BM memory T cell subsets are generated from the same immune responses, and suggest that in humans the CD69$^-$ BM memory CD4$^+$ T cells are not recirculating. Despite the presumed capacity of BM CD69$^-$ memory T cells to re-circulate (due to their lack of expression of CD69 and therefore expression of S1PR1), these cells seem to resemble to the BM CD69$^+$ cell population and reside preferentially in the tissue, separated from the circulating pool. It is not clear whether the expression of CD69 distinguishes two distinct BM populations, or if BM memory T cells are able to up- and down-regulate the expression of CD69.

Finally, these findings also suggest that $CD69^+$ and $CD69^-$ memory T cells are capable to provide systemic immunological protection, as they both maintain specificities for different antigens. In order to provide systemic immunity, $CD69^+$ memory T cells should be able to down-regulate CD69 to leave the BM and enter blood circulation or to divide in the BM after antigen encounter generating a progeny of $CD69^-$ daughter cells which could be able to enter blood circulation and confer fast immune protection.

4.2.2 Differences in TCRβ repertoire demonstrates compartmentalization of PB circulating and BM resident memory T cells

While assessing antigen specificities provides a measure of how the TCR repertoire is distributed in different sites, TCR sequencing provides a direct measure of individual T cell clone distribution, expansion and diversity. The TCR repertoire reflects the multiplicity of T lymphocytes, which are able to recognize a multitude of different antigens through highly variable αβ heterodimeric surface receptors. It has been estimated that over 2.5 x 10^7 unique TCRs are present in periphery [10,227]. Applying deep sequencing approaches, it is possible to survey all possible TCR rearrangements[228] and quantitatively assess the tissue compartmentalization of T cells. TCR analysis of T_{EM} cell sorted from spleen and two peripheral lymph nodes from individual donors revealed that the majority of $CD4^+$ T_{EM} in lymphoid tissue Ire unique to each site, with only some overlap between sites, while $CD8^+$ T_{EM} in lymphoid tissue showed increased sharing of clonally expanded populations between sites[102]. TCR analyses from tissue samples from patients afflicted with different diseases also revealed distinct repertoires in circulation and in different tissues. For example, T cells infiltrating colorectal tumors contained a distinct, non-overlapping repertoire compared to T cells in the neighboring mucosal tissue[229]. Together, these data suggest specific recruitment and/or retention of clonally expanded populations in different tissues. To further corroborate that PB and BM memory T cells also represent different populations strictly compartmentalized in their respective tissue, I compared the CDR3 TCRβ repertoire of PB and BM memory T cells by flow cytometric analysis and NGS.

Flow cytometric analysis of 24 TCR Vβ clones showed a highly diversified T cell repertoire in both PB and BM cell populations, containing all TCR Vβ families analyzed. The composition of TCR Vβ-chain families was very similar between $CD69^+$ and $CD69^-$ BM cell subsets, further suggesting that both memory T cell subsets are closely related. On the

other hand, differences in enrichment of some clones were detected when comparing BM to PB memory CD4+ T cells, further suggesting that cells from different compartments (PB circulating and BM resident) are preferentially enriched for different specificities. This flow cytometric approach using antibodies against 24 Vβ families identified in humans (which represent up to 70% of the whole Vβ families[181]) gives only a quick overview of the proportional TCR Vβ usage on a per-cell basis. However, this did not account for the plethora of individual clones within each family. NGS approaches allowed us to identify and quantitate each unique TCR clone in each sample. For this reason, I performed NGS of the TCRβ region and analyzed the individual CDR3 sequences of memory T cells from PB and BM.

First of all, analysis of technical replicates from the same sample showed low overlap in CD4+ (~35%), but was high for CD8+ memory T cells (>90%). Previous studies had shown that that the TCRβ estimated diversity among CD4+ T cells is ~5 times greater than in CD8+ T cells [230]. Therefore, the broad CDR3 TCRβ diversity present in all CD4+ memory T cell subsets and the limitations of the starting material (25000 sorted cells for each cell population) made it difficult to directly assess the complete repertoire. Based on simulations performed by Pawel Durek, I could estimate the overlap expected between technical replicates based on the Shannon Evenness Index (*SEI)* of the samples, which gives a direct measurement of the diversity found in each sample. Figure S6.1 from the appendices shows the relationship between the *SEI* and the percentage of overlap expected. CD4+ memory T cells presented a *SEI* of 0.96-0.98, which would correspond to an overlap of approximately 30%, whereas the overlap expected from memory CD8+ T cells (with and *SEI* of 0.18-0.43) would be of ~90%. These results indicate that low overlaps found between technical replicates of CD4+ memory T cells was not due to lack of robustness and reproducibility on the library preparation and sequencing processes, but due to other technical limitations present in our study. The determination of the sample size needed to assess a representative amount of TCRβ sequences would be of great importance in order to perform detailed analysis of the differences between circulating and tissue resident memory T cell populations.

Although it was difficult to know the exact numbers and frequencies of clonotypes shared between PB and BM memory T cells, I could make some conclusions using the available data. The analysis of similarities on the TCRβ repertoire between all samples showed no shared TCRβ chains between CD4+ and CD8+ memory T cells, as previously postulated[230].

When comparing frequencies of clones shared between the different $CD4^+$ memory T cell subsets I found low overlap between all three cell subsets (more than 70% of the sequences were unique for each cell subset). However, I found higher overlap of TCR clones between $CD69^+$ and $CD69^-$ BM memory T cells not found in their PB counterparts, suggesting that $CD69^+$ and $CD69^-$ memory T cells present in the BM come from same immune responses. On the other hand, $CD8^+$ memory T cells showed 50-70% overlap between the different T cell subsets, showing a closer relation of BM resident and PB circulating T cells. In contrast to $CD4^+$ memory T cells, I did not find major similarities between BM memory T cell subsets compared to their PB counterparts, suggesting that different specificities for $CD8^+$ memory T cells are maintained in a similar way between PB and BM cell subsets. So far, I have data showing that BM and PB memory $CD4^+$ T cell contain a different array of antigen specificities, with differences in frequencies and total numbers of antigen-specific memory T cells in both compartments[59]. However, the array of specificities of human PB and BM memory $CD8^+$ T cells has not been determined so far, leaving opened the possibility that both PB circulating and BM resident memory $CD8^+$ T cells share the same array of antigen specificities.

In order to perform a more specific analysis, I investigated the CDR3 TCRβ repertoire of TT-specific memory T cells isolated from PB and BM. Possibly due to repeated TT vaccinations throughout the adult life; TT-specific memory T cells are present in PB and BM, reason why I chose this antigen for our analysis. Results obtained showed low overlap (less than 25%) among TT-specific memory $CD4^+$ T cells from PB and BM, further indicating that PB and BM memory T cells are different populations separated and maintained in their respective compartment, with no or low interchange between them. Even when looking at cells specific to a same antigen, I could find differences in their TCRβ repertoire, suggesting that cells from the different compartments may react to different epitopes and may present differences in their affinity and potential response to the pathogens. Previous studies from our group showed higher frequencies and numbers of polyfunctional TT-specific memory T cells in the human BM compared to the PB counterparts[59], which could be related to the differences found on the TCRβ repertoire. Moreover, it would be interesting to perform further experiments using different dilutions of the antigens in order to see whether differences in the TCRβ repertoire are also associated with higher affinity in any of the two tissue compartments. One possibility could be that clonotypes found in the BM are the ones generated during the first infection or vaccination, which would be first recruited to the tissue to remain there for long periods,

whereas the ones in circulation are more recent clones generated during later vaccinations or infections. That could explain the fact that I only find antigen-specific memory CD4$^+$ T cells against childhood antigens in the BM of elderly donors but not in blood circulation, because cells generated with the vaccine would be recruited and maintained in the BM and slowly disappear from the blood circulation[38].

Until now, TCRβ repertoire analysis of humans has mostly been performed using blood samples, other organs and tissues being mostly neglected. However, recent studies have shown that T_{RM} cells seem to reside in their respective tissues without exchange with circulating cells, resulting in different pools of memory T cells stored in different parts of the human body. It would be interesting to determine the TCR repertoire in different tissues like BM, skin, gut, lungs, liver and also lymph nodes in order to cover the full TCR repertoire present in the human body and establish the differences between circulating and T_{RM} cells. This analysis would allow to determining whether specific clonotypes are preferentially enriched in particular tissues. Moreover, functional analysis of T_{RM} cells regarding their capacity to respond to different antigens compared with PB memory T cells would provide a better understanding of the significance of T_{RM} cells and T cell responses before, during and after infection or vaccination [231].

4.3 Mobilization of tissue resident memory CD4$^+$ T cells to blood circulation after systemic immune re-challenge

In humans, T cell responses (especially by memory CD4$^+$ T cells) after boost vaccination are poorly understood. In this study, I performed a longitudinal analysis of MMR-reactive memory CD4$^+$ T cell kinetics in donors in which pre-existing MMR-specific memory CD4$^+$ T cells was very reduced.

Measles, mumps and rubella viruses, are a highly infectious RNA virus that infects humans through the respiratory tract, resulting in a systemic immune response in the host and long-life antiviral immunity[187,188]. However, the mechanism underlying the protective immunity is not fully understood. Besides humoral immunity, it is believed that strong T-cell responses are important for antiviral immunity[39]. Memory CD4$^+$ T cells are pivotal in controlling humoral and cellular responses, therefore their longevity and response to vaccination are critical for the maintenance of protective immunity.

Previous work of our group and others have demonstrated that memory T cells are compartmentalized in tissues as T_{RM} cells with regard to their distinct functional properties. For example, it has been shown in both mice and man that, BM is home to memory T cells that are non-proliferative and sessile, have globally down regulated gene expression, and maintain functional long-term systemic memory[59,90,209]. In humans, $CD4^+$ memory T cells specific for viral childhood antigens, like measles, mumps or rubella have been found in the bone marrow of elderly, but not among circulating memory T cells[59]. These initial findings provoked the questions of whether and how T_{RM} cells respond to antigenic re-challenges.

Recent studies have shown that T_{RM} can respond rapidly to pathogen challenge at their local sites and mount local secondary immune reactions to provide faster protection at the site of infection [82,232]. Moreover, even though T_{RM} and T_{CM} seem to be generated from common clonal progenitors, T_{RM} cells of the skin mediated faster and more efficient responses after antigen re-challenge after intradermal immunization[233]. Most investigations performed on T_{RM} cells are focused on responses to local antigens at the site of infections, without looking at the responses that those cells could generate in the periphery. In contrast to the role of T_{RM} cells in other tissues -to provide local protection at sites of pathogen entry- BM memory T cells are thought to contribute to systemic memory[59]. However, the contribution of those cells to secondary immune response remains to be elucidated.

Early (i.e. before day 7) secondary $CD4^+$ cellular immune responses have never been investigated in detail before. I performed a study in which I analyzed the dynamics of antigen-reactive memory $CD4^+$ T cells at early time-points after immunization (16h as the earliest). I started with the assumption that, even when there are not circulating antigen-reactive memory T cells in blood; T_{RM} cells, such as the ones present in the BM, could be rapidly recruited into blood circulation and contribute to the secondary immune response.

For our study, I performed a screening of healthy volunteers in order to find those ones that had been in previous contact with at least one of the antigens (being positive for measles, mumps or rubella IgG-specific antibodies) but had low numbers of MMR-reactive memory $CD4^+$ T cells in blood circulation (because they have been vaccinated more than 30 years ago). From our screening of 24 healthy adults ranging from 24 to 43 years, I could only incorporate 11 suitable donors in our study. Moreover, due to strict regimens on

MMR vaccination in Germany, I could only find one donor which was naive for one of the antigens present in the vaccine.

4.3.1 Rapid and transient mobilization of antigen-reactive memory CD4⁺ T cells after MMR systemic immune re-challenge

Addressing MMR-reactive CD4⁺ T cells by stimulation assay and ICS revealed a typical anti-viral response [234], dominated by T_H1 cells expressing mostly IFNγ and TNFα [235]. After MMR vaccination, I found a rapid increase of MMR-reactive memory T cells in blood circulation, followed by a decrease at days 2-3 and a greater increase at days 7 and 14 after immunization. The rapid increase of non-proliferating MMR-reactive memory CD4⁺ T cells after vaccination (compared to the pre-existing circulating ones), suggest their mobilization from other tissues, such as the BM. In all cases analyzed, measles, mumps or rubella memory T cells were low in blood circulation before MMR vaccination, suggesting their preferential maintenance in other parts of the human body. The enrichment of measles, mumps and rubella (MMR)-specific memory CD4⁺ T cells in the BM [59] leads us to the assumption that the mobilized cells appearing in the blood circulation may have their origins from the quiescent pool previously found in BM. However, so far no other tissues have been analyzed for the presence of memory CD4⁺ T cells holding these specificities, so it is not possible to assure that the cells are uniquely coming from this tissue.

Interestingly, early appearing antigen-reactive memory T cells were mostly polyfunctional, expressing 2 or 3 cytokines at the same time. Previous studies from our group had shown that BM memory CD4⁺ T cells are mainly polyfunctional, further suggesting that mobilized cells may come from this tissue[59]. Polyfunctionality is proposed to be associated with protective immunity against various pathogens [189–191], producing more cytokine per cell compared to the single-producing ones and showing higher expression of CD154, which provides a better co-stimulation to CD8⁺ T and B cells[60]. Therefore, mobilized CD4⁺ memory T cells show high protective potential.

Moreover, early mobilized CD4⁺ memory T cells show a T_{RM}/ T_{EM} phenotype, with decreased expression of CCR7 on their surface. Non-lymphoid T_{EM} cells have more robust IFNγ production than T_{CM} cells, providing a mechanism to rapidly respond to re-infections. Similarly, CD4⁺ T cells that express CCR7 produce high amounts of IL-2 but low levels of other effector cytokines[236]. This effector memory phenotype goes along with the kinetics of

cytokine-producing memory T cells, suggesting that after vaccination, there is a transient mobilization of antigen-specific memory CD4$^+$ T cells resembling T_{RM}/T_{EM} phenotype, which are able to produce high amounts of effector-cytokines.

More importantly, early mobilized MMR-reactive memory CD4$^+$ T cells show no signs of proliferation, indicating that the increase of antigen-reactive memory CD4$^+$ T cells in blood after MMR vaccination is not due to proliferation of pre-existing memory T cells, but because of the mobilization of those cells from their site of residence. In support to our findings, a study analyzing the antigen responsiveness of naive and memory T cells using traceable TCR-transgenic T cells showed that memory T cells are quicker to respond but divide slow like naive cells. Both naive and memory T cells showed an approximate time delay of 3 days after infection, before they initiate cell division[237]. Altogether, these results indicate that antigen-reactive CD4$^+$ T cells can be reactivated in the tissue[238] and mobilized into the blood, from where they could migrate to secondary lymphoid organs or inflamed tissues.

After the early peak, the numbers of antigen-reactive memory CD4$^+$ T cells in blood circulation declined in the following 24-48 h, suggesting they may be already heading to the site of inflammation or to the secondary lymphoid organs. 7 and 14 days after vaccination, the frequencies and numbers of antigen-reactive memory CD4$^+$ T cells increased again in blood, probably reflecting the termination of the immune reaction and the egress of previously mobilized and newly formed memory T cells into the blood. This later influx of antigen-reactive CD4$^+$ T cells may be a mixture of pre-existing and newly generated effector memory CD4$^+$ T cells as a consequence of vaccination. As shown before for the early mobilized cells, late appearing antigen-reactive memory CD4$^+$ T cells are also mainly polyfunctional, suggesting their high protective potential, and show an effector memory phenotype. In contrast, the later accumulation of antigen-reactive memory CD4$^+$ T cells contains a high percentage of Ki-67$^+$ cells, suggesting they have undergone clonal expansion. Newly recruited cells might contribute to the heterogeneity and survival of the memory T cell pool. Ultimately, those memory T cells generated as a result of the MMR vaccination would remain in blood circulation for some time [239], but also relocate again in the BM, as evident from the previous analysis performed in elderly donors [59].

In contrast to the cellular kinetics of a secondary immune response observed in experienced donors, the analysis of a measles-naive donor showed a complete different scenario. No antigen-reactive memory T cells were detectable until day 14 after

vaccination, when both cytokine-producing and Ki-67$^+$ antigen-reactive CD4$^+$ T cells appeared in the bloodstream. In addition, antigen-reactive cells appearing at day 14 were mostly single-producers (~70%), which goes in line with reduced protective capacity of newly generated antigen-reactive T cells[240]. Interestingly, the measles-naive donor´s responses to mumps and rubella after MMR vaccination followed typical secondary immune responses, demonstrating a singular and specific immune response to each of the antigens present in the triple vaccine. The analysis of a primary response to the MMR vaccine provided us with information about the specificity of the system, but further naive subjects should be studied in order to verify the obtained results.

Altogether, results obtained from this analysis show that T_{RM} cells can be reactivated in the tissue[238] and mobilized into the blood, from where they could migrate to secondary lymphoid organs or inflamed tissues. Mobilized cells have high protective potential, mainly expressing 2 or 3 of the analyzed cytokines, and may contribute to create an effective response to the infectious agents. It is thought, that recall immune responses are mainly mediated by T_{CM} cells that patrol the T cell areas of secondary lymphoid organs where they can rapidly proliferate in response to antigens presented by dendritic cells (DC) [240]. However, here I show that T_{RM} cells mobilized from tissues can also mediate rapid and enhanced secondary immune responses.

4.3.2 Significant fraction of early mobilized antigen-reactive memory CD4$^+$ T cells present a T_{RM}/T_{EM} phenotype, with high expression of CD127

In order to better characterize the mobilized antigen-reactive memory T cells, I determined the expression of several cell lineage markers in antigen-reactive memory CD4$^+$ T cells.

The expression of CD137, CXCR5 and PD-1 expression on antigen-specific memory CD4$^+$ T cells showed no hint of mobilization of regulatory or follicular T cells. In concordance with the analysis of expression of CCR7 and cytokines in antigen-reactive memory CD4$^+$ T cells, mobilized cells show mainly a T_{RM}/T_{EM} phenotype, suggesting mainly cells with effector functions are mobilized in response to the antigen re-challenge.

Moreover, I determined the expression of the IL-7Rα (CD127) in antigen-reactive memory CD4$^+$ T cells. High expression of the IL-7α receptor (CD127) and the anti-apoptotic BCL-2 have been associated to enhanced survival capacities, and tend to decrease in recently generated effector CD4$^+$ T cells [198]. In our case, I found that early appearing MMR-specific

memory CD4$^+$ T cells maintained high levels of CD127 expression, whereas there was a reduction on the expression at days 7 and 14 after MMR vaccination. This data suggests that early appearing antigen-reactive memory CD4$^+$ T cells are most likely pre-existing memory T cells mobilized from their tissue of residence, and that they maintain high survival capacities. However, a fraction of antigen-reactive cells appearing at later time-points (days 7 and 14) would be generated as a result of activation and generation of new effector cells, thus having decreased expression of CD127.

Finally, in order analyze whether MMR-specific memory CD4$^+$ T cells were mobilized from the site of vaccine injection, I analyzed the expression of the skin homing marker CLA in the antigen-reactive cells. MMR-vaccination is administered subcutaneously, so it could be possible that after immunization, a pool of memory T cells are maintained as skin resident memory T cells at the site of injection. Those cells could then be the ones mobilized into blood circulation. I did not observe any drastic increase in CLA expression on antigen-reactive memory CD4$^+$ T cells, suggesting they are not mobilized from there. However, I only had the chance to perform this analysis in one subject, so further analysis should be done in order to verify this initial finding.

It remains to be clarified where early mobilized antigen-reactive memory CD4$^+$ T cells come from and where they are heading after they are firstly mobilized to the bloodstream. The lack of markers to identify BM memory T cells makes it impossible to determine from where they are coming from and where are they going. In order to address this question, a defined phenotype of T_{RM} cells from each tissue would be needed. However, T_{RM} cells could change their phenotype during the egress into the bloodstream, so it would be required to define those cells based in more stable characteristics like their DNA methylation status.

4.3.3 Bystander mobilization of TT-specific memory CD4$^+$ T cells in response to MMR vaccination

Human studies analyzing the dynamics of memory T cell responses to TT booster vaccination have observed that the expected expansion of TT-specific memory T cells was accompanied by an increase of memory T cells specific for two unrelated and not cross-reactive antigens: purified protein derivative from tuberculin (PPD) and Candida albicans, with similar kinetics to the specific response[199]. These bystander responses could result

from TCR-independent activation, most likely cytokine-mediated, occurring in a shared microenvironment during the vaccine-specific secondary immune response. In these studies, the $CD4^+$ memory T cell response was analyzed at later time after boost (1-2 weeks after vaccination) and followed until 16 weeks after. In this case, I was mainly interested in knowing whether early bystander mobilization also occurs as a result of MMR vaccination. Similar to the results obtained looking at the later response; I could also detect an early bystander mobilization of unrelated antigen-specific memory T cells in the blood, with similar kinetics to the ones observed in MMR-specific responses. Bystander mobilized TT-reactive memory $CD4^+$ T were also polyfunctional in terms of cytokine secretion, mainly producing TNFα and IL-2. In terms of immune protection, the ability of the mobilized bystander population to secrete pro-inflammatory cytokines at the site of infection would enhance the effectiveness of the specific response.

In order to clarify the nature of the bystander activated cells and the mechanisms driving their increase in peripheral blood, I addressed their phenotypic and functional features, comparing them with those of MMR-specific memory $CD4^+$ T cells. Previous studies have shown that vaccine-specific and bystander activated memory $CD4^+$ T cells differ in their proliferative status, as well as their expression of markers such as CCR7 and CD127 1-2 weeks after boost vaccination[200]. In our analysis, I wanted to study whether I could confirm these differences by looking at the MMR vaccine response, phenotypically characterizing vaccine-reactive and bystander mobilized memory $CD4^+$ T cells. Analysis of MMR-reactive memory T cells at days 7 and 14 after MMR vaccination showed similar results to the ones obtained in the TT vaccination study, mainly presenting an effector memory phenotype ($CCR7^-$), decreased CD127 expression and proliferating status ($Ki\text{-}67^+$). Bystander mobilized memory $CD4^+$ T cells maintained the frequencies of T_{CM} phenotype compared to before vaccination (40-50%), retained high expression of CD127 (80-90%) and were not proliferating, suggesting they are not recently activated effector cells, but they seem to derive from a central memory compartment[200]. Looking at the early mobilized cells (16 h and day 1), I did not detect any significant differences between vaccine-specific and bystander mobilized memory $CD4^+$ T cells. Both of them were non-proliferating cells with high expression of CD127. I could only detect an increase on the T_{RM}/T_{EM} memory T cell phenotype among MMR-reactive memory $CD4^+$ T cells, which was not observed among bystander-mobilized TT-specific memory $CD4^+$ T cells. Altogether, these results suggest that vaccination results in early mobilization and migration into the bloodstream of both, vaccine-specific and unrelated memory $CD4^+$ T cells. The contribution of the bystander

mobilized cells to the secondary immune response, as well as the reason for it remains to be elucidated.

4.3.4 MMR vaccine induces specific cellular and humoral responses

To verify whether early mobilized antigen-reactive memory $CD4^+$ T cells were specific for the vaccine antigens or cross-reactive to other antigens (present or not present in the vaccine), I isolated measles- and TT-specific $CD4^+CD54RA^-CD154^+CD69^+$ at day 1 after MMR vaccination, expanded them for 14 days [202] and re-stimulated them with vaccine-related and –unrelated antigens. Measles-reactive expanded cells reacted only to re-stimulation with measles antigen by up-regulating CD154 and producing several cytokines. Interestingly, cytokine producing cells maintained their polyfunctional capacities, with higher expression of IFNγ and TNFα. On the other hand, TT-specific memory $CD4^+$ T cells responded to TT re-stimulation but also reacted to other antigens such as measles or CMV, suggesting they were cross-reactive to other antigens.

Moreover, I followed the kinetics of the humoral immune response by analyzing the levels of IgG and IgM-specific antibodies. In all donors and specificities analyzed, I found an increase on IgG-specific antibodies at day 14 after vaccination, with no changes on the IgM levels. This response is the typical for a secondary immune response and shows a clear relation between the T and B cell immune responses. The maintenance of the levels of TT-specific neutralizing IgG antibodies during the time of the study, demonstrated that the antibody response generated by the MMR vaccination is specific for the vaccine antigens. While bystander $CD4^+$ memory T cells can secrete cytokines, and could potentially influence B cells, no increase in bystander antibody was evident in any of the vaccinees. These results suggest that antibody responses could be only generated in the presence of the antigen, and that in contrast to memory $CD4^+$ T cells, there is no bystander activation of B cells.

4.3.5 Mobilization of CD4$^+$ T_{RM} cells provides new functional TCR CDR3 clonotypes, which contribute to the immune response

The lack of proper cell surface markers identifying cells from a given compartment makes it very difficult to identify from where are mobilized antigen-reactive CD4$^+$ memory T cells. As the TCR is a unique identifier for specific T cell clones, I tracked specific T cells by their CDR3 TCRβ sequences. The TCR formation is not submitted to somatic hypermutation, so after the recombination process a unique CDR3 sequence is created and transmitted to the progeny through all its lifetime. Taking into account this information and previous results showing that different tissue compartments (PB and BM) show different TCR family repertoires in a global view and also at the level of antigen-specific CD4$^+$ memory T cells, I aimed to determine whether the early mobilized antigen-reactive memory T cells contained different CDR3 TCRβ clonotypes compared to the ones present in blood circulation before vaccination. Moreover; I aimed to know whether the newly appearing clonotypes as a result of vaccination would contribute to the immune response by being also present in blood at day 14 after immunization.

In our experiment, I used RNA rather than DNA since it allows straightforward introduction of Unique Molecular Identifiers (UMI), and also because it detects only the functional TCR molecules that are transcribed in memory CD4$^+$ T cells. Moreover, the use of RNA increases the likely coverage of the repertoire, since each cell can contain several molecules of messenger RNA. Even though this method is potentially sensitive to changes in RNA content associated with T cell activation and differentiation, several studies have demonstrated that such changes are small and transitory, and are unlikely to have a major impact in the repertoire[241,242]. Therefore, I could discard this bias of differential RNA expression upon activation via MMR vaccination.

MMR vaccination resulted in an influx of new clonal diversities, shown by the increase of the number of different clonotypes found among the same number of analyzed cells (2500 measles-reactive memory CD4$^+$ T cells). I aimed to know whether newly appearing CDR3 clonotypes at day 1 (not present in blood before MMR vaccination), further contributed to the immune response by being also present at day 14. Performing this analysis in 2 different donnors, I detected 6 and 5 abundant clonotypes respectively, which appeared at day 1 after vaccination and were still present at day 14. Of these, 3 clonotypes were expanded at day 14, whereas the rest were maintained at similar frequencies. On the other hand, none of the clones present before and at days 1 and 14 after vaccination were

expanded at day 14 compared with day 0 and 1. This data suggests that T_{RM} $CD4^+$ cells rapidly mobilized after MMR vaccination play a role in the generation of the secondary immune response, even to a greater extent than pre-existing memory $CD4^+$ T cells in circulation.

In order to determine whether the newly appearing cells are coming from the BM, it would be really informative to have sequences from the TCRβ clonotypes present in the antigen-specific memory $CD4^+$ T cells from paired PB and BM prior to vaccination. However, patients undergoing hip replacement operations (source of our samples) are mainly elderly donors, so it would be not permitted to perform the MMR vaccination after the operation. Moreover, I do not know if other tissues share the same clonotype diversity than the one found in the BM, so it would not be possible to confirm that cells are coming from the BM.

4.4 Concluding remarks

For a long time, maintenance of long-term T cell memory has been a matter of extensive investigations and debate. After demonstrating that the human BM hosts long-lived memory T cells capable of providing systemic immunological protection, and resting in terms of activation, transcription and proliferation[59], a central question has been whether circulating and BM resident memory T cells differ in their maintenance, compartmentalization and function.

BM memory T cells are maintained in specific survival niches, where they are subjected to the tissue conditions and receiving signals needed for their long-term maintenance. In contrast, memory T cells present in blood are in constant circulation through blood, secondary lymphoid organs and tissues, fact that makes them more likely to adapt to different environmental conditions. Maintenance of BM memory T cells in a resting state would result in the requirement of fewer resources compared to the long-term maintenance of actively circulating memory T cells, and may be a mechanism of the human body to sustain a huge array of antigen specificities, even when the antigen is long past. Cytokines such as IL-7 and IL-15 as well as direct contact with stromal cells are required for memory T cells to be sustained in a resting state in the BM, while retaining their capacities to respond to reactivation signals an confer us with protection after systemic immune re-challenges. After systemic immune re-challenges with long-past antigens, such as MMR, BM memory $CD4^+$ T cells have the capacity to respond and be

mobilized into blood circulation in order to contribute to the generation of a highly protective secondary immune response

The knowledge obtained from this doctoral thesis provide a better understanding of the maintenance and function of immunological memory in humans, and could be used for the development of new vaccines and immunotherapies. Induction of a more prominent population of BM memory T cells could be an advantage for the prolonged maintenance of highly protective systemic memory. The enhancement of protective memory as well as depletion of pathological memory could be achieved by knowing the mechanisms involved in memory T cell survival.

5 References

1. Charles A Janeway, J., Travers, P., Walport, M. & Shlomchik, M. J. Principles of innate and adaptive immunity. (2001).
2. Spits, H. Development of αβ t cells in the human thymus. *Nat. Rev. Immunol.* **2,** 760–772 (2002).
3. Inaba, K., Metlay, J. P., Crowley, M. T., Witmer-Pack, M. & Steinman, R. M. Dendritic Cells as Antigen Presenting Cells *in Vivo*. *Int. Rev. Immunol.* **6,** 197–206 (1990).
4. Lo Presti, E., Dieli, F. & Meraviglia, S. Tumor-Infiltrating Î³Î´ T Lymphocytes: Pathogenic Role, Clinical Significance, and Differential Programing in the Tumor Microenvironment. *Front. Immunol.* **5,** 607 (2014).
5. Burtrum, D. B., Kim, S., Dudley, E. C., Hayday, A. C. & Petrie, H. T. TCR gene recombination and alpha beta-gamma delta lineage divergence: productive TCR-beta rearrangement is neither exclusive nor preclusive of gamma delta cell development. *J. Immunol.* **157,** 4293–6 (1996).
6. Miles, J. J., Douek, D. C. & Price, D. A. Bias in the αβ T-cell repertoire: implications for disease pathogenesis and vaccination. *Immunol. Cell Biol.* **89,** 375–387 (2011).
7. Turner, S. J., Doherty, P. C., McCluskey, J. & Rossjohn, J. Structural determinants of T-cell receptor bias in immunity. *Nat. Rev. Immunol.* **6,** 883–894 (2006).
8. Robins, H. S. *et al.* Overlap and effective size of the human CD8 + T-cell receptor repertoire. doi:10.1126/scitranslmed.3001442
9. Murugan, A., Mora, T., Walczak, A. M. & Callan, C. G. Statistical inference of the generation probability of T-cell receptors from sequence repertoires.
10. Arstila, T. P. *et al.* A Direct Estimate of the Human αβ T Cell Receptor Diversity. *Science (80-.).* **286,** (1999).
11. Warren, R. L. *et al.* Exhaustive T-cell repertoire sequencing of human peripheral blood samples reveals signatures of antigen selection and a directly measured repertoire size of at least 1 million clonotypes. *Genome Res.* **21,** 790–7 (2011).
12. Wan, Y. Y. Multi-tasking of helper T cells. *Immunology* **130,** 166–71 (2010).
13. Mullen, A. C. *et al.* Role of T-bet in Commitment of TH1 Cells Before IL-12-Dependent Selection. *Science (80-.).* **292,** 1907–1910 (2001).
14. Szabo, S. J. *et al.* A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* **100,** 655–69 (2000).
15. Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136,** 2348–57 (1986).
16. Heinzel, F. P., Sadick, M. D., Holaday, B. J., Coffman, R. L. & Locksley, R. M. Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J. Exp. Med.* **169,** 59–72 (1989).
17. Taylor-Robinson, A. W., Phillips, R. S., Severn, A., Moncada, S. & Liew, F. Y. The role of TH1 and TH2 cells in a rodent malaria infection. *Science* **260,** 1931–4 (1993).
18. Taylor-Robinson, A. W. & Phillips, R. S. Functional characterization of protective CD4+ T-cell clones reactive to the murine malaria parasite Plasmodium chabaudi. *Immunology* **77,** 99–105 (1992).
19. Dardalhon, V. *et al.* IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat. Immunol.* **9,** 1347–55 (2008).
20. Veldhoen, M. *et al.* Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat. Immunol.* **9,** 1341–6 (2008).
21. Goswami, R. & Kaplan, M. H. A brief history of IL-9. *J. Immunol.* **186,** 3283–8 (2011).

22. Zheng, W. & Flavell, R. A. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* **89,** 587–96 (1997).

23. Huang, W., Na, L., Fidel, P. L. & Schwarzenberger, P. Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. *J. Infect. Dis.* **190,** 624–31 (2004).

24. Infante-Duarte, C., Horton, H. F., Byrne, M. C. & Kamradt, T. Microbial lipopeptides induce the production of IL-17 in Th cells. *J. Immunol.* **165,** 6107–15 (2000).

25. Mangan, P. R. *et al.* Transforming growth factor-β induces development of the TH17 lineage. *Nature* **441,** 231–234 (2006).

26. Yang, X. O. *et al.* T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* **28,** 29–39 (2008).

27. Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M. & Stockinger, B. TGFβ in the Context of an Inflammatory Cytokine Milieu Supports De Novo Differentiation of IL-17-Producing T Cells. *Immunity* **24,** 179–189 (2006).

28. Puel, A. *et al.* Autoantibodies against IL-17A, IL-17F, and IL-22 in patients with chronic mucocutaneous candidiasis and autoimmune polyendocrine syndrome type I. *J. Exp. Med.* **207,** 291–297 (2010).

29. Liu, X. *et al.* Bcl6 expression specifies the T follicular helper cell program in vivo. *J. Exp. Med.* **209,** 1841–52, S1-24 (2012).

30. Breitfeld, D. *et al.* Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J. Exp. Med.* **192,** 1545–52 (2000).

31. Hori, S., Nomura, T. & Sakaguchi, S. Control of Regulatory T Cell Development by the Transcription Factor Foxp3. *Science (80-.).* **299,** 1057–1061 (2003).

32. Moore, K. W. *et al.* Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. *Science* **248,** 1230–4 (1990).

33. Mittrücker, H.-W., Visekruna, A. & Huber, M. Heterogeneity in the Differentiation and Function of CD8+ T Cells. *Arch. Immunol. Ther. Exp. (Warsz).* **62,** 449–458 (2014).

34. Panum, P. L. Observations made during the epidemic of measles on the Faroe Islands in the year 1846. *Chall. Epidemiol. Issues Sel. Readings, Pan Am. Heal. Organ. New York* 37–41 (1988). doi:10.2105/AJPH.30.10.1245

35. Harrington, L. E., Most Rv, R. van der, Whitton, J. L. & Ahmed, R. Recombinant vaccinia virus-induced T-cell immunity: quantitation of the response to the virus vector and the foreign epitope. *J. Virol.* **76,** 3329–37 (2002).

36. Crotty, S. *et al.* Cutting Edge: Long-Term B Cell Memory in Humans after Smallpox Vaccination. *J. Immunol.* **171,** (2003).

37. Combadiere, B. *et al.* Distinct time effects of vaccination on long-term proliferative and IFN-gamma-producing T cell memory to smallpox in humans. *J. Exp. Med.* **199,** 1585–93 (2004).

38. Hammarlund, E. *et al.* Duration of antiviral immunity after smallpox vaccination. *Nat. Med.* **9,** 1131–7 (2003).

39. van Els, C. A. C. M. & Nanan, R. T Cell Responses in Acute Measles. *Viral Immunol.* **15,** 435–450 (2002).

40. Sant, A. J. *et al.* Immunodominance in CD4 T-cell responses: implications for immune responses to influenza virus and for vaccine design. *Expert Rev. Vaccines* **6,** 357–368 (2007).

41. Wrammert, J. & Ahmed, R. Maintenance of serological memory. *Biol. Chem.* **389,** 537–9 (2008).

42. Slifka, M. K., Antia, R., Whitmire, J. K. & Ahmed, R. Humoral Immunity Due to Long-Lived Plasma Cells. *Immunity* **8,** 363–372 (1998).

43. Hou, S., Hyland, L., Ryan, K. W., Portner, A. & Doherty, P. C. Virus-specific CD8+ T-cell memory determined by clonal burst size. *Nature* **369,** 652–654 (1994).

44. Sabbagh, L. *et al.* The Selective Increase in Caspase-3 Expression in Effector but Not Memory T Cells Allows Susceptibility to Apoptosis. *J. Immunol.* **173,** (2004).

45. Diaz-Guerra, E., Vernal, R., del Prete, M. J., Silva, A. & Garcia-Sanz, J. A. CCL2 Inhibits the Apoptosis Program Induced by Growth Factor Deprivation, Rescuing Functional T Cells. *J. Immunol.* **179,** (2007).

46. Kaech, S. M. & Ahmed, R. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naïve cells. *Nat. Immunol.* **2,** 415–422 (2001).

47. Kaech, S. M. & Wherry, E. J. Heterogeneity and cell-fate decisions in effector and memory CD8+ T cell differentiation during viral infection. *Immunity* **27,** 393–405 (2007).

48. Surh, C. D., Boyman, O., Purton, J. F. & Sprent, J. Homeostasis of memory T cells. *Immunol. Rev.* **211,** 154–163 (2006).

49. Hu, H. *et al.* CD4(+) T cell effectors can become memory cells with high efficiency and without further division. *Nat. Immunol.* **2,** 705–10 (2001).

50. Wherry, E. J. *et al.* Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat. Immunol.* **4,** 225–234 (2003).

51. Farber, D. L. Cutting Edge Commentary: Differential TCR Signaling and the Generation of Memory T Cells. *J. Immunol.* **160,** (1998).

52. Chang, J. T. *et al.* Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science* **315,** 1687–1691 (2007).

53. Woodland, D. L. Cell-mediated immunity to respiratory virus infections. *Curr. Opin. Immunol.* **15,** 430–435 (2003).

54. Dutton, R. W., Bradley, L. M. & Swain, S. L. T CELL MEMORY. *Annu. Rev. Immunol.* **16,** 201–223 (1998).

55. Busch, D. H. & Pamer, E. G. T cell affinity maturation by selective expansion during infection. *J. Exp. Med.* **189,** 701–10 (1999).

56. Slifka, M. K. & Whitton, J. L. Functional avidity maturation of CD8(+) T cells without selection of higher affinity TCR. *Nat. Immunol.* **2,** 711–7 (2001).

57. Turula, H., Smith, C. J., Grey, F., Zurbach, K. A. & Snyder, C. M. Competition between T cells maintains clonal dominance during memory inflation induced by MCMV. *Eur. J. Immunol.* **43,** 1252–63 (2013).

58. Kumar, R. *et al.* Increased Sensitivity of Antigen-Experienced T Cells through the Enrichment of Oligomeric T Cell Receptor Complexes. *Immunity* **35,** 375–387 (2011).

59. Okhrimenko, A. *et al.* Human memory T cells from the bone marrow are resting and maintain long-lasting systemic memory. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 9229–34 (2014).

60. Kannanganat, S., Ibegbu, C., Chennareddi, L., Robinson, H. L. & Amara, R. R. Multiple-Cytokine-Producing Antiviral CD4 T Cells Are Functionally Superior to Single-Cytokine-Producing Cells. *J. Virol.* **81,** 8468–8476 (2007).

61. Sallusto, F., Lenig, D., Förster, R., Lipp, M. & Lanzavecchia, A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401,** 708–712 (1999).

62. Masopust, D., Vezys, V., Marzo, A. L. & Lefrançois, L. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* **291,** 2413–7 (2001).

63. Reinhardt, R. L., Khoruts, a, Merica, R., Zell, T. & Jenkins, M. K. Visualizing the generation of memory CD4 T cells in the whole body. *Nature* **410,** 101–105 (2001).

64. Gattinoni, L. *et al.* A human memory T cell subset with stem cell-like properties. *Nat Med* **17,** 1290–1297 (2011).

65. Luckey, C. J. & Weaver, C. T. Stem-cell-like qualities of immune memory; CD4+ T cells join the party. *Cell Stem Cell* **10,** 107–8 (2012).

66. Fuertes Marraco, S. A. *et al.* Long-lasting stem cell–like memory CD8+ T cells with a naïve-like profile upon yellow fever vaccination. *Sci. Transl. Med.* **7,** (2015).

67. Takeshita, M. *et al.* Polarization diversity of human CD4+ stem cell memory T cells. *Clin. Immunol.* **159,** 107–117 (2015).

68. Lugli, E. *et al.* Superior T memory stem cell persistence supports long-lived T cell memory. *J. Clin. Invest.* **123,** 594–9 (2013).

69. Schenkel, J. M., Fraser, K. A. & Masopust, D. Cutting edge: resident memory CD8 T cells occupy frontline niches in secondary lymphoid organs. *J. Immunol.* **192,** 2961–4 (2014).

70. Masopust, D. *et al.* Dynamic T cell migration program provides resident memory within intestinal epithelium. *J. Exp. Med.* **207,** (2010).

71. Steinert, E. M. *et al.* Quantifying Memory CD8 T Cells Reveals Regionalization of Immunosurveillance. *Cell* **161,** 737–49 (2015).

72. Clark, R. A. *et al.* The Vast Majority of CLA+ T Cells Are Resident in Normal Skin. *J. Immunol.* **176,** (2006).

73. Clark, R. A. *et al.* A Novel Method for the Isolation of Skin Resident T Cells from Normal and Diseased Human Skin. *J. Invest. Dermatol.* **126,** 1059–1070 (2006).

74. Purwar, R. *et al.* Resident memory T cells (T(RM)) are abundant in human lung: diversity, function, and antigen specificity. *PLoS One* **6,** e16245 (2011).

75. Ganusov, V. V. & De Boer, R. J. Do most lymphocytes in humans really reside in the gut? *Trends Immunol.* **28,** 514–518 (2007).

76. Di Rosa, F. & Pabst, R. The bone marrow: a nest for migratory memory T cells. *Trends Immunol.* **26,** 360–366 (2005).

77. Tokoyoda, K. *et al.* Professional Memory CD4+ T Lymphocytes Preferentially Reside and Rest in the Bone Marrow. *Immunity* **30,** 721–730 (2009).

78. Herndler-Brandstetter, D. *et al.* Human bone marrow hosts polyfunctional memory CD4+ and CD8+ T cells with close contact to IL-15-producing cells. *J. Immunol.* **186,** 6965–71 (2011).

79. Gebhardt, T. *et al.* Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat. Immunol.* **10,** 524–530 (2009).

80. Hofmann, M., Oschowitzer, A., Kurzhals, S. R., Krüger, C. C. & Pircher, H. Thymus-resident memory CD8 $^{+}$ T cells mediate local immunity. *Eur. J. Immunol.* **43,** 2295–2304 (2013).

81. Wakim, L. M., Woodward-Davis, A. & Bevan, M. J. Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 17872–9 (2010).

82. Jiang, X. *et al.* Skin infection generates non-migratory memory CD8+ TRM cells providing global skin immunity. *Nature* **483,** 227–231 (2012).

83. Mackay, L. K. *et al.* Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. *Proc. Natl. Acad. Sci. U. S. A.* **109,** 7037–42 (2012).

84. Shin, H. & Iwasaki, A. A vaccine strategy that protects against genital herpes by establishing local memory T cells. *Nature* **491,** 463–7 (2012).

85. Chang, J. T., Wherry, E. J. & Goldrath, A. W. Molecular regulation of effector and memory T cell differentiation. *Nat. Rev. Immunol.* **15,** 1104–1115 (2014).

86. Kaech, S. M. & Cui, W. Transcriptional control of effector and memory CD8+ T cell differentiation. *Nat. Rev. Immunol.* **12,** 749–61 (2012).

87. Mackay, L. K. *et al.* The developmental pathway for CD103+CD8+ tissue-resident memory T cells of skin. **14,** 1294–1301 (2013).

88. Mackay, L. K. *et al.* Cutting Edge: CD69 Interference with Sphingosine-1-Phosphate Receptor Function Regulates Peripheral T Cell Retention. *J. Immunol.* **194,** (2015).

89. Ely, K. H., Cookenham, T., Roberts, A. D. & Woodland, D. L. Memory T Cell Populations in the Lung Airways Are Maintained by Continual Recruitment. *J. Immunol.* **176,** (2005).

90. Tokoyoda, K. *et al.* Professional Memory CD4+ T Lymphocytes Preferentially Reside and Rest in the Bone Marrow. *Immunity* **30,** 721–730 (2009).

91. Laidlaw, B. J. *et al.* CD4+ T cell help guides formation of CD103+ lung-resident memory CD8+ T cells during influenza viral infection. *Immunity* **41,** 633–45 (2014).

92. Zhang, N. & Bevan, M. J. Transforming growth factor-β signaling controls the formation and maintenance of gut-resident memory T cells by regulating migration and retention. *Immunity* **39,** 687–96 (2013).

93. Casey, K. A. *et al.* Antigen-independent differentiation and maintenance of effector-like resident memory T cells in tissues. *J. Immunol.* **188,** 4866–75 (2012).

94. Cepek, K. L. *et al.* Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the αEβ7 integrin. *Nature* **372,** 190–193 (1994).

95. Ray, S. J. *et al.* The collagen binding alpha1beta1 integrin VLA-1 regulates CD8 T cell-mediated immune protection against heterologous influenza infection. *Immunity* **20,** 167–79 (2004).

96. Wakim, L. M. *et al.* The molecular signature of tissue resident memory CD8 T cells isolated from the brain. *J. Immunol.* **189,** 3462–71 (2012).

97. Cyster, J. G. & Schwab, S. R. Sphingosine-1-Phosphate and Lymphocyte Egress from Lymphoid Organs. *Annu. Rev. Immunol.* **30,** 69–94 (2012).

98. Masopust, D., Vezys, V., Wherry, E. J., Barber, D. L. & Ahmed, R. Cutting edge: gut microenvironment promotes differentiation of a unique memory CD8 T cell population. *J. Immunol.* **176,** 2079–83 (2006).

99. Skon, C. N. Transcriptional downregulation of *S1pr1* is required for the establishment of resident memory CD8 T cells. *Nat. Immunol.* (2013). doi:10.1038/ni.2745

100. Bai, A., Hu, H., Yeung, M. & Chen, J. Kruppel-like factor 2 controls T cell trafficking by activating L-selectin (CD62L) and sphingosine-1-phosphate receptor 1 transcription. *J. Immunol.* **178,** 7632–9 (2007).

101. Sathaliyawala, T. *et al.* Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets. *Immunity* **38,** 187–97 (2013).

102. Thome, J. J. C. *et al.* Spatial map of human T cell compartmentalization and maintenance over decades of life. *Cell. Novemb.* **6,** 814–828 (2014).

103. Vezys, V. *et al.* Memory CD8 T-cell compartment grows in size with immunological experience. *Nature* **457,** 196–199 (2009).

104. Badovinac, V. P., Haring, J. S. & Harty, J. T. Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection. *Immunity* **26,** 827–41 (2007).

105. Woodland, D. L. & Kohlmeier, J. E. Migration, maintenance and recall of memory T cells in peripheral tissues. *Nat. Rev. Immunol.* **9,** 153–61 (2009).

106. Bergsbaken, T. & Bevan, M. J. Proinflammatory microenvironments within the intestine regulate the differentiation of tissue-resident $CD8^+$ T cells responding to infection. *Nat. Immunol.* **16,** 406–14 (2015).

107. Schenkel, J. M., Fraser, K. A., Vezys, V. & Masopust, D. Sensing and alarm function of resident memory CD8+ T cells. *Nat. Immunol.* **14,** 509–513 (2013).

108. Glennie, N. D. *et al.* Skin-resident memory CD4+ T cells enhance protection against Leishmania major infection. *J. Exp. Med.* **212,** 1405–14 (2015).

109. Vukmanovic-Stejic, M. *et al.* Varicella zoster-specific CD4+Foxp3+ T cells accumulate after cutaneous antigen challenge in humans. *J. Immunol.* **190,** 977–86 (2013).

110. de Bree, G. J. *et al.* Characterization of $CD4^+$ Memory T Cell Responses Directed against Common Respiratory Pathogens in Peripheral Blood and Lung. *J. Infect. Dis.* **195,** 1718–1725 (2007).

111. de Bree, G. J. *et al.* Selective accumulation of differentiated CD8+ T cells specific for respiratory viruses in the human lung. *J. Exp. Med.* **202,** 1433–42 (2005).

112. Travlos, G. S. Normal structure, function, and histology of the bone marrow. *Toxicol. Pathol.* **34,** 548–65 (2006).

113. Benner, R., Meima, F. & van der Meulen, G. M. Antibody formation in mouse bone marrow. II. Evidence for a memory-dependent phenomenon. *Cell. Immunol.* **13,** 95–106 (1974).

114. McHeyzer-Williams, L. J. & McHeyzer-Williams, M. G. ANTIGEN-SPECIFIC MEMORY B CELL DEVELOPMENT. *Annu. Rev. Immunol.* **23,** 487–513 (2005).

115. Ueda, Y., Kondo, M. & Kelsoe, G. Inflammation and the reciprocal production of granulocytes and lymphocytes in bone marrow. *J. Exp. Med.* **201,** 1771–1780 (2005).

116. Walsh, M. C. *et al.* OSTEOIMMUNOLOGY: Interplay Between the Immune System and Bone Metabolism. *Annu. Rev. Immunol.* **24,** 33–63 (2006).

117. Prockop, D. J. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276,** 71–4 (1997).

118. Zeng, D. *et al.* Unique patterns of surface receptors, cytokine secretion, and immune functions distinguish T cells in the bone marrow from those in the periphery: impact on allogeneic bone marrow transplantation. *Blood* **99,** (2002).

119. Westermann, J. & Pabst, R. Distribution of lymphocyte subsets and natural killer cells in the human body. *Clin. Investig.* **70,** 539–44 (1992).

120. Schenkel, J. M. & Masopust, D. Tissue-resident memory T cells. *Immunity* **41,** 886–97 (2014).

121. von Andrian, U. H. & Mackay, C. R. T-cell function and migration. Two sides of the same coin. *N. Engl. J. Med.* **343,** 1020–34 (2000).

122. Koni, P. A. *et al.* Conditional vascular cell adhesion molecule 1 deletion in mice: impaired lymphocyte migration to bone marrow. *J. Exp. Med.* **193,** 741–54 (2001).

123. Mazo, I. B. *et al.* Bone Marrow Is a Major Reservoir and Site of Recruitment for Central Memory CD8+ T Cells. *Immunity* **22,** 259–270 (2005).

124. Avigdor, A. *et al.* CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow. *Blood* **103,** (2004).

125. Maeda, Y., Seki, N., Sato, N., Sugahara, K. & Chiba, K. Sphingosine 1-phosphate receptor type 1 regulates egress of mature T cells from mouse bone marrow. *Int. Immunol.* **22,** 515–525 (2010).

126. Bankovich, A. J., Shiow, L. R. & Cyster, J. G. CD69 suppresses sphingosine 1-phosophate receptor-1 (S1P1) function through interaction with membrane helix 4. *J. Biol. Chem.* **285,** 22328–22337 (2010).

127. Carlson, C. M. *et al.* Kruppel-like factor 2 regulates thymocyte and T-cell migration. *Nature* **442,** 299–302 (2006).

128. Shinoda, K. *et al.* Type II membrane protein CD69 regulates the formation of resting T-helper memory. *Proc. Natl. Acad. Sci. U. S. A.* **109,** 7409–14 (2012).

129. Hanazawa, A. *et al.* CD49b-dependent establishment of T helper cell memory. *Immunol. Cell Biol.* **91,** 524–531 (2013).

130. Feuerer, M. *et al.* Bone marrow as a priming site for T-cell responses to blood-borne antigen. **9,** 1151–1157 (2003).

131. Letsch, A. *et al.* CMV-specific central memory T cells reside in bone marrow. *Eur. J. Immunol.* **37,** 3063–3068 (2007).

132. Palendira, U. *et al.* Selective accumulation of virus-specific CD8+ T cells with unique homing phenotype within the human bone marrow. *Blood* **112,** 3293–3302 (2008).

133. Guerreiro, M. *et al.* Human peripheral blood and bone marrow Epstein-Barr virus-specific T-cell repertoire in latent infection reveals distinct memory T-cell subsets. *Eur. J. Immunol.* **40,** 1566–1576 (2010).

134. Casorati, D. M. *et al.* bone marrow transplantation recipients. antigen-experienced CD4+ T cells in allogeneic persistence of donor-derived Restricted TCR repertoire and long-term Restricted TCR Repertoire and Long-Term Persistence of Donor-Derived Antigen-Experienced CD4+ T Cells in Allogeneic Bone Marrow Transplantation Recipients'. *J Immunol* **157,** 5739–5747 (1996).

135. Sercan Alp, Ö. *et al.* Memory CD8(+) T cells colocalize with IL-7(+) stromal cells in bone marrow and rest in terms of proliferation and transcription. *Eur. J. Immunol.* **45,** 975–87 (2015).

136. Tokoyoda, K., Egawa, T., Sugiyama, T., Choi, B.-I. & Nagasawa, T. Cellular Niches Controlling B Lymphocyte Behavior within Bone Marrow during Development. *Immunity* **20,** 707–718 (2004).

137. Khaled, A. R. & Durum, S. K. Lymphocide: cytokines and the control of lymphoid homeostasis. *Nat Rev Immunol.* **2,** 817–830 (2002).

138. Witte Heather A Minges Wols, P. L., Underhill, G. H., Minges Wols, H. A., Kansas, G. S. & Witte, P. L. Longevity Cells in the Maintenance of Plasma Cell The Role of Bone Marrow-Derived Stromal The Role of Bone Marrow-Derived Stromal Cells in the Maintenance of Plasma Cell Longevity. *J Immunol Ref.* **169,** 4213–4221 (2002).

139. Manz Muehlinghaus, R. A. *et al.* Adhesion-Dependent Signals Synergistic Effects of Cytokines and Plasma Cell Survival Is Mediated by Plasma Cell Survival Is Mediated by Synergistic Effects of Cytokines and Adhesion-Dependent Signals. *J Immunol Ref.* **171,** 1684–1690 (2003).

140. Ma, A., Koka, R. & Burkett, P. Diverse Functions of IL-2, IL-15, and IL-7 in Lymphoid Homeostasis. *Annu. Rev. Immunol* **24,** 657–79 (2006).

141. Rochman, I., Paul, W. E. & Ben-Sasson, S. Z. Survival IL-6 Increases Primed Cell Expansion and IL-6 Increases Primed Cell Expansion and Survival. *J Immunol Ref.* **174,** 4761–4767 (2005).

142. Giron-Michel, J. *et al.* Differential STAT3, STAT5, and NF-κB activation in human hematopoietic progenitors by endogenous interleukin-15: implications in the expression of functional molecules. *Blood* **102,** (2003).

143. Fehniger, T. A. & Caligiuri, M. A. Interleukin 15: biology and relevance to human disease. *Blood* **97,** (2001).

144. Tan, J. T. *et al.* Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. *J. Exp. Med.* **195,** 1523–32 (2002).

145. Schluns, K. S., Kieper, W. C., Jameson, S. C. & Lefrançois, L. Interleukin-7 mediates the homeostasis of na|[iuml]|ve and memory CD8 T cells in vivo. *Nat. Immunol.* **1,** 426–432 (2000).

146. Lefrançois Kimberly Schluns, L. S. *et al.* Antigen-Specific CD8 T Cells Generation of Primary and Memory Cutting Edge: Requirement for IL-15 in the Cutting Edge: Requirement for IL-15 in the Generation of Primary and Memory Antigen- Specific CD8 T Cells 1. *J Immunol Ref.* **168,** 4827–4831 (2002).

147. Purton, J. F. *et al.* Antiviral CD4+ memory T cells are IL-15 dependent. *J. Exp. Med.* **204,** 951–61 (2007).

148. Kondrack, R. M. *et al.* Interleukin 7 regulates the survival and generation of memory CD4 cells. *J. Exp. Med.* **198,** 1797–1806 (2003).

149. Zhang, X., Sun, S., Hwang, I., Tough, D. F. & Sprent, J. Potent and Selective Stimulation of Memory-Phenotype CD8. *Immunity* **8,** 591–599 (1998).

150. Goldrath, A. W. *et al.* Cytokine Requirements for Acute and Basal Homeostatic Proliferation of Naive and Memory CD8+ T Cells. *J. Exp. Med.* **195,** (2002).

151. Judge, A. D., Zhang, X., Fujii, H., Surh, C. D. & Sprent, J. Interleukin 15 Controls both Proliferation and Survival of a Subset of Memory-Phenotype CD8+ T Cells. *J. Exp. Med.* **196,** (2002).

152. Sudo, T. *et al.* Expression and function of the interleukin 7 receptor in murine lymphocytes. *Immunology* **90,** 9125–9129 (1993).

153. Tuckwell, D., Calderwood, D. A., Green, L. J. & Humphries, M. J. Integrin alpha 2 I-domain is a binding site for collagens. *J. Cell Sci.* **108,** (1995).

154. Heino, J. The collagen family members as cell adhesion proteins. *BioEssays* **29,** 1001–1010 (2007).

155. Shimizu, Y. *et al.* Four molecular pathways of T cell adhesion to endothelial cells: roles of LFA-1, VCAM-1, and ELAM-1 and changes in pathway hierarchy under different activation conditions. *J. Cell Biol.* **113,** 1203–12 (1991).

156. Ryan, D. H., Nuccie, B. L., Abboud, C. N. & Winslow, J. M. Vascular cell adhesion molecule-1 and the integrin VLA-4 mediate adhesion of human B cell precursors to cultured bone marrow adherent cells. *J. Clin. Invest.* **88,** 995–1004 (1991).

157. Wang, M. W. *et al.* Rescue from apoptosis in early (CD34-selected) versus late (non-CD34-selected) human hematopoietic cells by very late antigen 4- and vascular cell adhesion molecule (VCAM) 1-dependent adhesion to bone marrow stromal cells. *Cell Growth Differ.* **9,** 105–12 (1998).

158. DiLillo, D. J. *et al.* Maintenance of Long-Lived Plasma Cells and Serological Memory Despite Mature and Memory B Cell Depletion during CD20 Immunotherapy in Mice. *J. Immunol.* **180,** (2007).

159. Carreau, A., Hafny-Rahbi, B. El, Matejuk, A., Grillon, C. & Kieda, C. Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. *J. Cell. Mol. Med.* **15,** 1239–1253 (2011).

160. Chipuk, J. E. & Green, D. R. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? doi:10.1016/j.tcb.2008.01.007

161. van Delft, M. F. & Huang, D. C. How the Bcl-2 family of proteins interact to regulate apoptosis. *Cell Res.* **16,** 203–213 (2006).

162. Giam, M., Huang, D. & Bouillet, P. BH3-only proteins and their roles in programmed cell death. *Oncogene* **27,** 128–136 (2009).

163. Certo, M. *et al.* Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* **9,** 351–65 (2006).

164. Chen, L. *et al.* Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol. Cell* **17,** 393–403 (2005).

165. Kim, H. *et al.* Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. *Nat. Cell Biol.* **8,** 1348–1358 (2006).

166. Grad, J. M., Zeng, X. R. & Boise, L. H. Regulation of Bcl-xL: a little bit of this and a little bit of STAT. *Curr. Opin. Oncol.* **12,** 543–9 (2000).

167. Zhong, Q., Gao, W., Du, F. & Wang, X. Mule/ARF-BP1, a BH3-Only E3 Ubiquitin Ligase, Catalyzes the Polyubiquitination of Mcl-1 and Regulates Apoptosis. *Cell* **121,** 1085–1095 (2005).

168. Oda, E. *et al.* Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288,** 1053–8 (2000).

169. Alves, N. L. *et al.* The Noxa/Mcl-1 Axis Regulates Susceptibility to Apoptosis under Glucose Limitation in Dividing T Cells. *Immunity* **24,** 703–716 (2006).

170. Kim, J.-Y., Ahn, H.-J., Ryu, J.-H., Suk, K. & Park, J.-H. BH3-only Protein Noxa Is a Mediator of Hypoxic Cell Death Induced by Hypoxia-inducible Factor 1 ␣. *J. Exp. Med. J. Exp. Med* **11131100,** 113–123 (2004).

171. Bouillet, P. *et al.* Proapoptotic Bcl-2 Relative Bim Required for Certain Apoptotic Responses, Leukocyte Homeostasis, and to Preclude Autoimmunity. *Science (80-.).* **286,** (1999).

172. Veis, D. J., Sorenson, C. M., Shutter, J. R. & Korsmeyer, S. J. Bcl-2-Deficient Mice Demonstrate Fulminant Lymphoid Apoptosis, Polycystic Kidneys, and Hypopigmented Hair. *Cell* **75,** 229–240 (1993).

173. Opferman, J. T. *et al.* Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. *Nature* **426,** 671–6 (2003).

174. Dunkle, A., Dzhagalov, I. & He, Y.-W. Cytokine-dependent and cytokine-independent roles for Mcl-1: genetic evidence for multiple mechanisms by which Mcl-1 promotes survival in primary T lymphocytes. *Cell Death Dis.* **2,** (2011).

175. Nijhawan, D. *et al.* Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. *Genes Dev.* **17,** 1475–86 (2003).

176. Yamashita, M. *et al.* Bmi1 regulates memory CD4 T cell survival via repression of the *Noxa* gene. *J. Exp. Med.* (2008). doi:10.1084/jem.20072000

177. Foxwell, B. M. J., Beadling, C., Guschin, D., Kerr, I. & Cantrell, D. Interleukin-7 can induce the activation of Jak 1, Jak 3 and STAT 5 proteins in murine T cells. *Eur. J. Immunol.* **25,** 3041–3046 (1995).

178. Pernis, A. *et al.* Gamma chain-associated cytokine receptors signal through distinct transducing factors. *J. Biol. Chem.* **270,** 14517–22 (1995).

179. Johnson, S. E., Shah, N., Bajer, A. A. & LeBien, T. W. IL-7 Activates the Phosphatidylinositol 3-Kinase/AKT Pathway in Normal Human Thymocytes but Not Normal Human B Cell Precursors. *J. Immunol.* **180,** (2008).

180. Chetoui, N., Boisvert, M., Gendron, S. & Aoudjit, F. Interleukin-7 promotes the survival of human CD4 $^{+}$ effector/memory T cells by up-regulating Bcl-2 proteins and activating the JAK/STAT signalling pathway. *Immunology* **130,** 418–426 (2010).

181. Clark, S. P., Arden, B., Kabelitz, D. & Mak, T. W. Comparison of human and mouse T-cell receptor variable gene segment subfamilies. *Immunogenetics* **42,** 531–40 (1995).

182. Cossarizza, A. *et al.* Guidelines for the use of flow cytometry and cell sorting in immunological studies. *Eur. J. Immunol.* **47,** 1584–1797 (2017).

183. Sasson, S. C. *et al.* Increased Plasma Interleukin-7 Level Correlates with Decreased CD127 and Increased CD132 Extracellular Expression on T Cell Subsets in Patients with HIV-1 Infection. *J. Infect. Dis.* **193,** 505–514 (2006).

184. Colle, J.-H. *et al.* CD127 expression and regulation are altered in the memory CD8 T cells of HIV-infected patients - reversal by highly active anti-retroviral therapy (HAART). *Clin. Exp. Immunol.* **143,** 398–403 (2006).

185. Kivioja, T. *et al.* Counting absolute numbers of molecules using unique molecular identifiers. *Nat. Methods* **9,** 72–74 (2011).

186. Hill, M. O. Diversity and Evenness: A Unifying Notation and Its Consequences. *Ecology* **54,** 427–432 (1973).

187. Amanna, I. J., Carlson, N. E. & Slifka, M. K. Duration of Humoral Immunity to Common Viral and Vaccine Antigens. *N. Engl. J. Med.* **357,** 1903–1915 (2007).

188. Lambert, N., Strebel, P., Orenstein, W., Icenogle, J. & Poland, G. A. Rubella. *Lancet (London, England)* **385,** 2297–307 (2015).

189. Darrah, P. A. *et al.* Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat. Med.* **13,** 843–850 (2007).

190. Seder, R. A., Darrah, P. A. & Roederer, M. T-cell quality in memory and protection: implications for vaccine design. *Nat. Rev. Immunol.* **8,** 247–258 (2008).

191. Boyd, A. *et al.* Pathogen-Specific T Cell Polyfunctionality Is a Correlate of T Cell Efficacy and Immune Protection. *PLoS One* **10,** e0128714 (2015).

192. Miller, J. D. *et al.* Human Effector and Memory CD8+ T Cell Responses to Smallpox and Yellow Fever Vaccines. *Immunity* **28,** 710–722 (2008).

193. Schulz, A. R. *et al.* Low Thymic Activity and Dendritic Cell Numbers Are Associated with the Immune Response to Primary Viral Infection in Elderly Humans. *J. Immunol.* **195,** 4699–711 (2015).

194. Gerdes, S. J. *et al.* Cell cycle analysis of a cell CELL CYCLE ANALYSIS OF A CELL PROLIFERATION-ASSOCIATED HUMAN NUCLEAR ANTIGEN DEFINED BY THE MONOCLONAL ANTIBODY Ki-67'. *J Immunol J. Immunol.* **133,** 1710–1715 (1984).

195. Chakrabarti, L. A. *et al.* Normal T-cell turnover in sooty mangabeys harboring active simian immunodeficiency virus infection. *J. Virol.* **74,** 1209–23 (2000).

196. Mueller, S. N., Gebhardt, T., Carbone, F. R. & Heath, W. R. Memory T Cell Subsets, Migration Patterns, and Tissue Residence. *Annu. Rev. Immunol.* **31,** 137–161 (2013).

197. Lefrançois, L. & Masopust, D. T cell immunity in lymphoid and non-lymphoid tissues. *Curr. Opin. Immunol.* **14,** 503–508 (2002).

198. Cellerai, C., Harari, A., Vallelian, F., Boyman, O. & Pantaleo, G. Functional and phenotypic characterization of tetanus toxoid-specific human CD4± T cells following re-immunization. *Eur. J. Immunol.* **37,** 1129–1138 (2007).

199. Di Genova, G., Roddick, J., McNicholl, F. & Stevenson, F. K. Vaccination of human subjects expands both specific and bystander memory T cells but antibody production remains vaccine specific. *Blood* **107,** 2806–13 (2006).

200. Li Causi, E. *et al.* Vaccination Expands Antigen-Specific CD4+ Memory T Cells and Mobilizes Bystander Central Memory T Cells. *PLoS One* **10,** e0136717 (2015).

201. Bacher, P. *et al.* Antigen-Reactive T Cell Enrichment for Direct, High-Resolution Analysis of the Human Naive and Memory Th Cell Repertoire. *J. Immunol.* **190,** (2013).

202. Scheffold Kniemeyer, A. *et al.* Repertoire Human Naive and Memory Th Cell Direct, High-Resolution Analysis of the Antigen-Reactive T Cell Enrichment for Antigen-Reactive T Cell Enrichment for Direct, High-Resolution Analysis of the Human Naive and Memory Th Cell Repertoire. *J Immunol Mater. Suppl.* **190,** 3967–3976 (2013).

203. Fisher, R. A., Corbet, A. S. & Williams, C. B. The Relation Between the Number of Species and the Number of Individuals in a Random Sample of an Animal Population. *J. Anim. Ecol.* **12,** 42 (1943).

204. EFRON, B. & THISTED, R. Estimating the number of unseen species: How many words did Shakespeare know? *Biometrika* **63,** 435–447 (1976).

205. Robins, H. S. *et al.* Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. *Blood* **114,** 4099–107 (2009).

206. Zvyagin, I. V *et al.* Distinctive properties of identical twins' TCR repertoires revealed by high-throughput sequencing. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 5980–5 (2014).

207. Butcher, E. C. & Picker, L. J. Lymphocyte homing and homeostasis. *Science* **272,** 60–6 (1996).

208. Tokoyoda, K., Hauser, A. E., Nakayama, T. & Radbruch, A. Organization of immunological memory by bone marrow stroma. *Nat. Rev. Immunol.* **10,** 193–200 (2010).

209. Sercan Alp, Ö. *et al.* Memory CD8 $^{+}$ T cells colocalize with IL-7 $^{+}$ stromal cells in bone marrow and rest in terms of proliferation and transcription. *Eur. J. Immunol.* **45,** 975–987 (2015).

210. Shiow, L. R. *et al.* CD69 acts downstream of interferon-α/β to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* **440,** 540–544 (2006).

211. Biancotto, A. *et al.* Baseline Levels and Temporal Stability of 27 Multiplexed Serum Cytokine Concentrations in Healthy Subjects. *PLoS One* **8,** e76091 (2013).

212. Hotamisligil, G. S. & Bernlohr, D. A. Metabolic functions of FABPs--mechanisms and therapeutic implications. *Nat. Rev. Endocrinol.* **11,** 592–605 (2015).

213. Pan, Y. *et al.* Survival of tissue-resident memory T cells requires exogenous lipid uptake and metabolism. *Nat. Publ. Gr.* **543,** (2017).

214. Becker, T. C., Coley, S. M., Wherry, E. J. & Ahmed, R. Bone marrow is a preferred site for homeostatic proliferation of memory CD8 T cells. *J. Immunol.* **174,** 1269–73 (2005).

215. Parretta, E. *et al.* CD8 cell division maintaining cytotoxic memory occurs predominantly in the bone marrow. *J.Immunol.* **174,** 7654–7664 (2005).

216. Siracusa, F. *et al.* Maintenance of CD8 $^{+}$ memory T lymphocytes in the spleen but not in the bone marrow is dependent on proliferation. *Eur. J. Immunol.* **47,** 1900–1905 (2017).

217. Adachi, T. *et al.* Hair follicle-derived IL-7 and IL-15 mediate skin-resident memory T cell homeostasis and lymphoma. *Nat. Med.* **21,** 1272–9 (2015).

218. Chu, V. T. *et al.* Eosinophils are required for the maintenance of plasma cells in the bone marrow. *Nat. Immunol.* **12,** 151–159 (2011).

219. Jourdan, M. *et al.* IL-6 supports the generation of human long-lived plasma cells in combination with either APRIL or stromal cell-soluble factors. *Leukemia* **28,** 1647–1656 (2014).

220. El-Asady, R. *et al.* TGF-{beta}-dependent CD103 expression by CD8(+) T cells promotes selective destruction of the host intestinal epithelium during graft-versus-host disease. *J. Exp. Med.* **201,** 1647–57 (2005).

221. Skon, C. N. *et al.* Transcriptional downregulation of S1pr1 is required for the establishment of resident memory CD8+ T cells. *Nat. Immunol.* **14,** 1285–93 (2013).

222. Surh, C. D. & Sprent, J. Homeostasis of Naive and Memory T Cells. *Immunity* **29,** 848–862 (2008).

223. Zehentmeier, S. *et al.* Static and dynamic components synergize to form a stable survival niche for bone marrow plasma cells. *Eur. J. Immunol.* **44,** 2306–17 (2014).

224. Gottlieb, T. M. & Oren, M. p53 in growth control and neoplasia. *Biochim. Biophys. Acta* **1287,** 77–102 (1996).

225. Moll, U. M. & Zaika, A. Nuclear and mitochondrial apoptotic pathways of p53. *FEBS Lett.* **493,** 65–9 (2001).

226. Farber, D. L., Yudanin, N. A. & Restifo, N. P. Human memory T cells: generation, compartmentalization and homeostasis. *Nat. Rev. Immunol.* **14,** 24–35 (2014).

227. Davenport, M. P. *et al.* Pyrosequencing Cell Subsets and Individuals Revealed by A Mechanism for TCR Sharing between T. (2011). doi:10.4049/jimmunol.1003898

228. Robins, H. Immunosequencing: applications of immune repertoire deep sequencing. *Curr. Opin. Immunol.* **25,** 646–652 (2013).

229. Sherwood, A. M. *et al.* Tumor-infiltrating lymphocytes in colorectal tumors display a diversity of T cell receptor sequences that differ from the T cells in adjacent mucosal tissue. *Cancer Immunol. Immunother.* **62,** 1453–61 (2013).

230. Li, H. M. *et al.* TCRβ repertoire of CD4+ and CD8+ T cells is distinct in richness, distribution, and CDR3 amino acid composition. *J. Leukoc. Biol.* **99,** 505–13 (2016).

231. Woodsworth, D. J., Castellarin, M. & Holt, R. A. Sequence analysis of T-cell repertoires in health and disease. *Genome Med.* **5,** 98 (2013).

232. Clark, R. A. Resident memory T cells in human health and disease. *Sci. Transl. Med.* **7,** 269rv1 (2015).

233. Gaide, O. *et al.* Common clonal origin of central and resident memory T cells following skin immunization. *Nat. Med.* **21,** 647–653 (2015).

234. Swain, S. L., McKinstry, K. K. & Strutt, T. M. Expanding roles for CD4+ T cells in immunity to viruses. *Nat. Rev. Immunol.* (2012). doi:10.1038/nri3152

235. Kohler, S. *et al.* The early cellular signatures of protective immunity induced by live viral vaccination. *Eur. J. Immunol.* **42,** 2363–2373 (2012).

236. Mahnke, Y. D., Brodie, T. M., Sallusto, F., Roederer, M. & Lugli, E. The who's who of T-cell differentiation: Human memory T-cell subsets. *Eur. J. Immunol.* **43,** 2797–2809 (2013).

237. Whitmire, J. K., Eam, B. & Whitton, J. L. Tentative T cells: memory cells are quick to respond, but slow to divide. *PLoS Pathog.* **4,** e1000041 (2008).

238. Cavanagh, L. L. *et al.* Activation of bone marrow-resident memory T cells by circulating, antigen-bearing dendritic cells.

239. Amanna, I. J., Slifka, M. K. & Crotty, S. Immunity and immunological memory following smallpox vaccination. *Immunol. Rev.* **211,** 320–337 (2006).

240. Sallusto, F., Lanzavecchia, A., Araki, K. & Ahmed, R. From Vaccines to Memory and Back. *Immunity* **33,** 451 (2010).

241. Lindsten, T., June, C. H. & Thompson, C. B. Transcription of T cell antigen receptor genes is induced by protein kinase C activation. *J. Immunol.* **141,** 1769–74 (1988).

242. Paillard, F., Sterkers, G. & Vaquero, C. Transcriptional and post-transcriptional regulation of TcR, CD4 and CD8 gene expression during activation of normal human T lymphocytes. *EMBO J.* **9,** 1867–72 (1990).

6 Appendices

A

	memory CD4$^+$ CD69$^+$ BM				memory CD4$^+$ CD69$^-$ BM				memory CD4$^+$ CD69$^-$ PB			
	mean (%)	SD	min (%)	max (%)	mean (%)	SD	min (%)	max (%)	mean (%)	SD	min (%)	max (%)
Vβ1	2.923	0.494	2.370	3.810	3.205	0.879	2.160	5.140	3.414	1.161	1.720	5.410
Vβ2	8.461	1.543	6.000	10.200	8.174	1.212	6.600	9.510	7.865	1.416	4.870	9.540
Vβ3	4.274	2.637	1.050	8.030	4.033	2.404	1.330	7.320	4.008	2.470	1.050	7.340
Vβ4	2.448	0.744	1.390	3.690	2.579	0.505	2.080	3.380	2.624	0.747	2.010	4.350
Vβ5.1	5.404	2.419	1.690	9.970	5.063	2.583	1.290	9.980	8.206	7.847	1.020	26.200
Vβ5.2	0.738	0.419	0.251	1.500	0.869	0.679	0.302	2.380	0.844	0.459	0.340	1.880
Vβ5.3	0.950	0.875	0.314	3.060	1.158	1.137	0.286	3.860	1.113	1.033	0.498	3.640
Vβ7.1	1.530	0.893	0.301	3.270	1.376	0.896	0.250	3.280	1.755	0.834	1.040	3.470
Vβ7.2	1.983	1.654	0.870	5.520	1.725	1.812	0.589	5.740	1.884	2.224	0.540	6.830
Vβ8	7.119	5.204	2.790	19.300	5.889	4.188	2.920	14.500	8.848	12.100	2.450	38.500
Vβ9	2.530	1.805	0.922	6.680	2.455	1.774	1.070	6.680	1.684	0.779	0.565	3.070
Vβ11	0.932	1.192	0.220	3.820	1.037	1.233	0.160	3.930	0.779	0.613	0.274	2.220
Vβ12	2.426	1.721	1.280	5.680	1.990	1.319	0.946	5.150	1.445	0.490	0.834	1.990
Vβ13.1	2.993	1.931	1.180	7.120	3.456	2.601	1.990	9.780	3.076	2.198	1.310	8.350
Vβ13.2	2.011	0.740	0.730	3.100	1.779	0.714	0.460	2.740	2.085	0.966	0.720	3.680
Vβ13.6	1.843	0.694	1.140	3.290	1.651	0.536	1.020	2.330	1.667	0.733	0.840	3.120
Vβ14	2.596	1.476	1.030	5.840	2.496	1.320	0.940	5.260	2.193	1.309	0.720	4.640
Vβ16	1.211	0.656	0.760	2.780	1.073	0.763	0.560	2.920	1.181	0.868	0.580	3.270
Vβ17	5.178	1.136	3.450	6.750	4.940	1.243	3.220	6.650	4.628	1.189	2.760	6.490
Vβ18	1.180	0.587	0.282	2.310	1.385	0.880	0.480	3.000	0.907	0.431	0.162	1.700
Vβ20	2.378	0.863	1.150	3.690	2.651	1.639	0.869	4.920	2.106	1.045	0.705	3.660
Vβ21.3	2.463	1.548	1.190	5.890	2.658	2.628	0.570	8.600	1.293	0.652	0.421	2.310
Vβ22	3.301	0.553	2.250	3.920	3.328	0.909	2.310	4.840	3.266	0.827	1.930	4.100
Vβ23	0.381	0.239	0.102	0.820	0.394	0.255	0.156	0.940	0.404	0.187	0.193	0.656

B

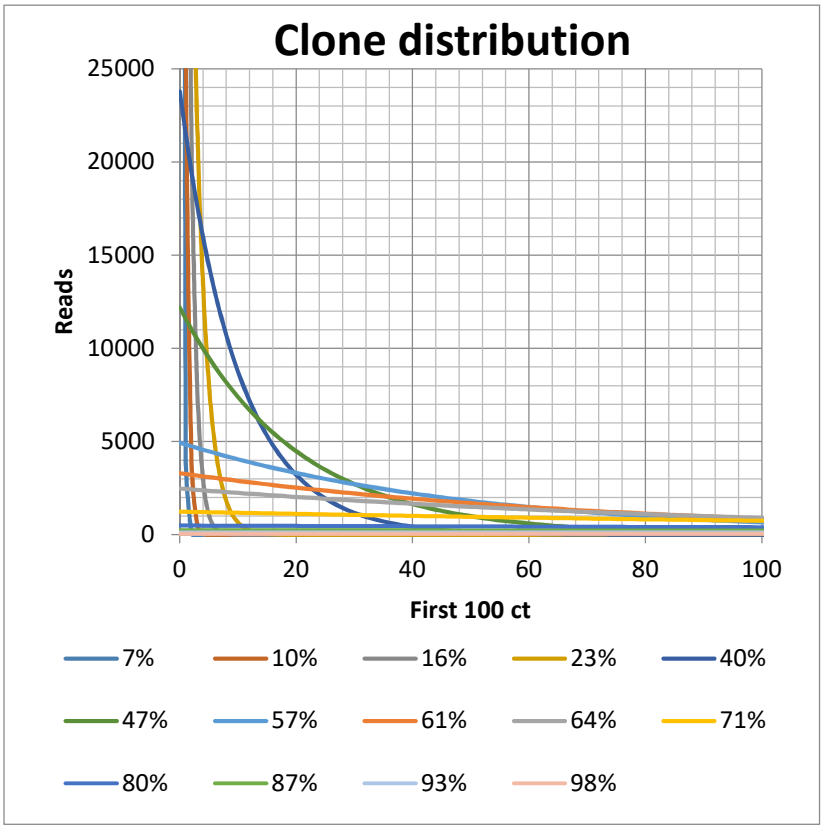
	memory CD8$^+$ CD69$^+$ BM				memory CD8$^+$ CD69$^-$ BM				memory CD8$^+$ CD69$^-$ PB			
	mean (%)	SD	min (%)	max (%)	mean (%)	SD	min (%)	max (%)	mean (%)	SD	min (%)	max (%)
Vβ1	3.444	1.559	1.280	5.790	3.376	0.655	2.430	4.130	4.153	1.516	2.400	7.550
Vβ2	8.916	8.811	1.910	29.100	6.395	4.412	1.300	16.000	6.936	5.762	1.430	18.600
Vβ3	4.103	4.735	0.123	14.900	4.279	3.391	0.560	10.600	3.899	3.198	0.203	9.770
Vβ4	1.873	1.053	0.720	3.660	2.071	1.089	0.390	3.780	3.069	1.319	1.000	5.030
Vβ5.1	3.429	3.585	0.341	11.400	2.818	1.696	0.566	5.420	3.761	3.472	0.247	10.600
Vβ5.2	0.639	0.728	0.069	2.340	0.875	0.825	0.349	2.820	0.747	0.705	0.150	2.400
Vβ5.3	1.410	1.108	0.174	3.380	1.494	1.914	0.246	6.070	1.459	1.785	0.087	5.380
Vβ7.1	1.497	0.779	0.747	3.270	2.211	0.524	1.370	2.770	2.335	0.780	1.500	4.040
Vβ7.2	2.067	0.801	1.370	3.830	2.216	1.339	0.940	4.700	3.075	4.534	0.790	13.300
Vβ8	3.284	4.527	0.680	14.300	4.910	5.346	1.550	17.700	10.820	20.710	1.440	61.800
Vβ9	1.176	0.636	0.259	2.230	1.300	0.588	0.386	2.270	0.779	0.421	0.253	1.370
Vβ11	0.775	1.248	0.124	3.790	1.141	1.552	0.177	4.760	0.933	1.260	0.186	3.890
Vβ12	1.395	1.394	0.526	4.670	2.007	1.695	0.930	6.030	1.987	1.961	0.561	6.600
Vβ13.1	4.055	3.796	0.668	11.600	2.903	1.899	0.677	5.910	3.018	2.289	0.450	7.620
Vβ13.2	2.620	2.010	0.604	5.150	3.098	2.677	0.785	8.090	4.160	5.196	0.670	13.500
Vβ13.6	3.295	5.378	0.460	16.300	2.655	2.680	0.830	8.690	1.838	1.799	0.613	6.050
Vβ14	6.019	6.537	1.410	21.600	5.258	2.474	1.780	8.970	5.405	2.417	2.080	9.060
Vβ16	1.313	0.582	0.435	1.980	1.329	0.530	0.580	2.270	0.850	0.280	0.490	1.330
Vβ17	4.715	3.343	0.970	11.400	4.303	1.936	2.590	8.520	4.459	2.246	2.790	9.720
Vβ18	0.868	1.442	0.077	4.070	0.605	0.739	0.017	2.010	0.553	0.547	0.098	1.720
Vβ20	1.066	0.428	0.649	2.000	1.897	0.770	0.868	3.170	1.783	1.727	0.675	5.860
Vβ21.3	1.651	1.083	0.671	3.610	1.615	0.643	0.767	2.510	1.137	0.511	0.351	1.950
Vβ22	1.846	1.238	0.791	4.760	2.738	0.906	1.280	3.900	3.435	1.920	1.190	6.080
Vβ23	3.136	4.193	0.062	11.000	1.960	1.848	0.223	5.500	1.298	0.704	0.320	2.560

Table 6-1: TCRβ repertoire usage of PB, BM CD69$^-$ and BM CD69$^+$ CD4$^+$ (A) and CD8$^+$ (B) memory T cells. Data presented as mean, standard deviation (SD), minimum and maximum values of each TCR Vβ family (n=8).

Sample	N°cells	Replicates		UMI counts		Clonotypes		SEI	
		A	B	A	B	A	B	A	B
$CD4^+CD45RA^-CD69^-$_PB	25000	½ RNA	½ RNA	5692	5396	4035	3911	0.9820	0.9834
$CD4^+CD45RA^-CD69^-$_BM	25000	½ RNA	½ RNA	2166	1830	1094	913	0.9604	0.9571
$CD4^+CD45RA^-CD69^+$_BM	25000	½ RNA	½ RNA	8017	4080	5293	2955	0.9789	0.9794
$CD8^+CD45RA^-CD69^-$_PB	25000	½ RNA	½ RNA	11644	12605	725	851	0.4093	0.4371
$CD8^+CD45RA^-CD69^-$_BM	25000	½ RNA	½ RNA	28887	31851	500	514	0.1874	0.2313
$CD8^+CD45RA^-CD69^+$_BM	25000	½ RNA	½ RNA	18307	16287	1070	988	0.3672	0.3608

Table 6-2: Number of cells sorted, UMI counts, clonotypes found and *SEI* among samples from which technical replicates were performed. Raw data is shown for both technical replicates of each sample analyzed.

A



B

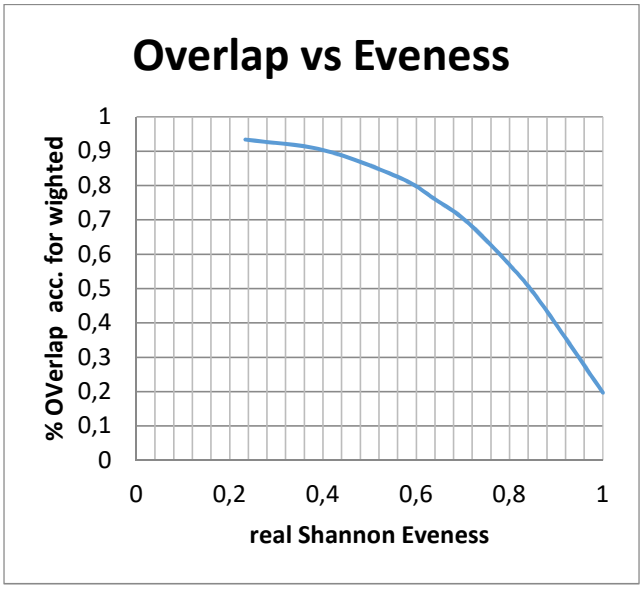
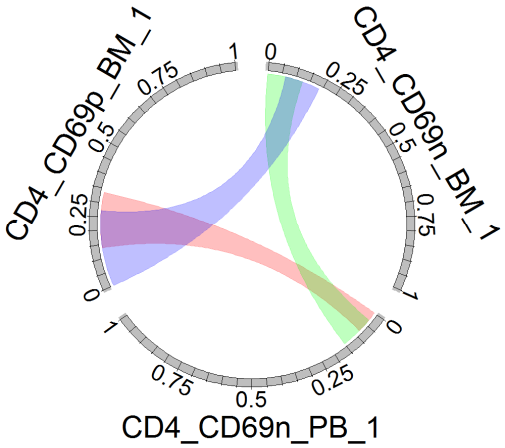


Figure 6-1: Simulations of the relationship between diversity and overlap between technical replicates. (A) Representation of the clonal distribution of the top 100 clones regarding the Shanon Eveness Index (SEI). Each line represents an established SEI, resulting in different clonal distributions. (B) Relationship between *SEI* and percentages of overlap expected between samples.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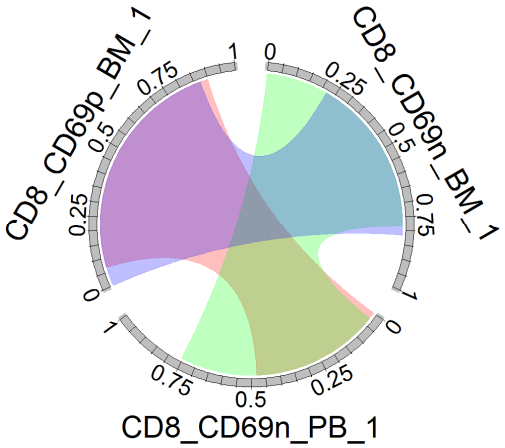
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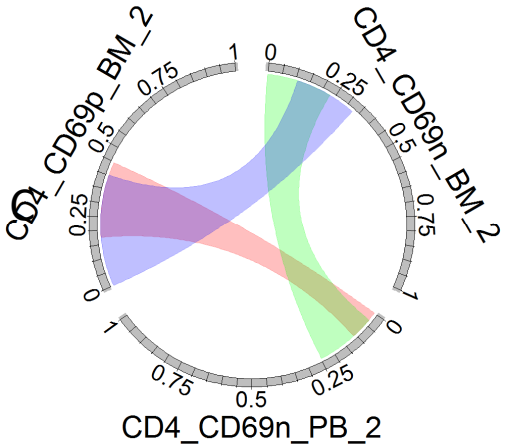
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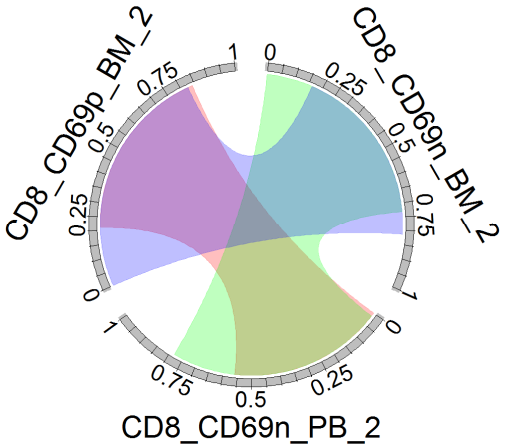
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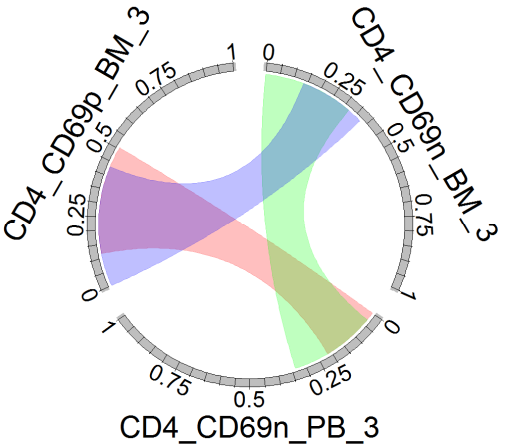
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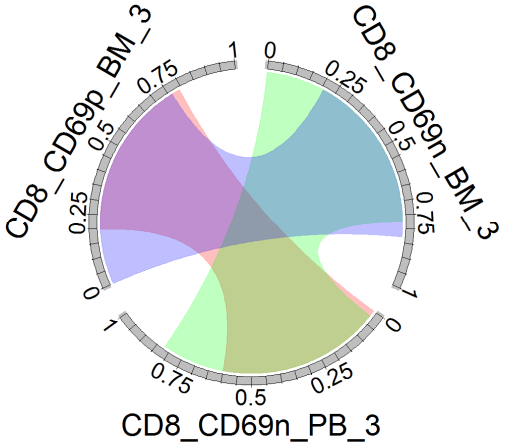
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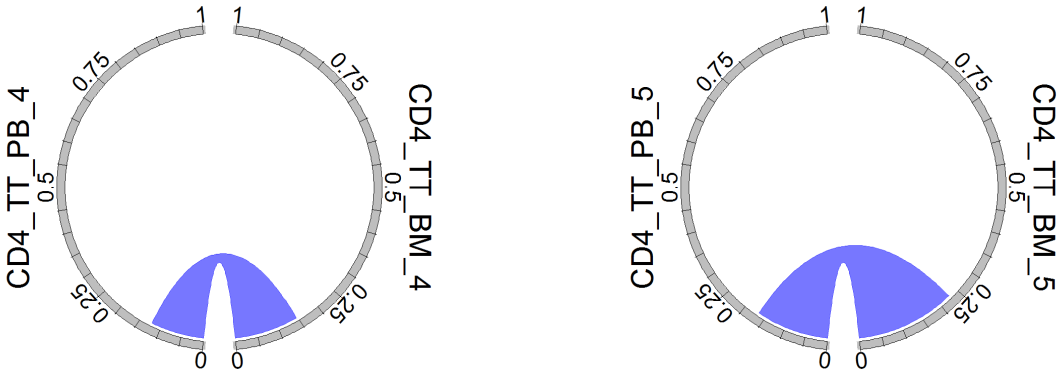

Figure 6-2: l space shared between memory T cell subsets from PB and BM samples of additional donors. ncing libraries were performed as mentioned before. CDR3 sequences obtained from replicates ooled (in cases were replicates were performed) and down-s d in order to compare the same amount of nces. Frequencies of the clonal space shared between different memory T cell subsets are represented as circular graphs (A) Frequencies of overlap between memory $CD4^+$ subsets from PB and BM samples, regarding CD69 expression (n=3) (B) Frequencies of overlap between memory $CD8^+$ subsets from PB and BM samples, regarding CD69 expression (n=3). (C) Frequencies of overlap between TT-specific memory $CD4^+$ cell subsets from PB and BM samples (n=2).

Donor	Gender	Age	First Ag. encounter	Analysis
MMR2	Female	34	MMR vaccination	Cellular analysis
MMR3	Female	32	MMR vaccination	Cellular analysis
MMR4	Female	29	Naïve measles	Cellular analysis
MMR5	Female	26	MMR vaccination	Cellular analysis
MMR7	Male	42	Natural infection	Cellular analysis
MMR8	Male	31	MMR vaccination	Cellular analysis
MMR10	Male	37	MMR vaccination	TCRβ sequencing
MMR11	Female	34	MMR vaccination	TCRβ sequencing

Table 6-3: Information of healthy volunteers re-vaccinated with the MMR vaccine. Gender, age, first antigen encounter and analysis performed is depicted in the table

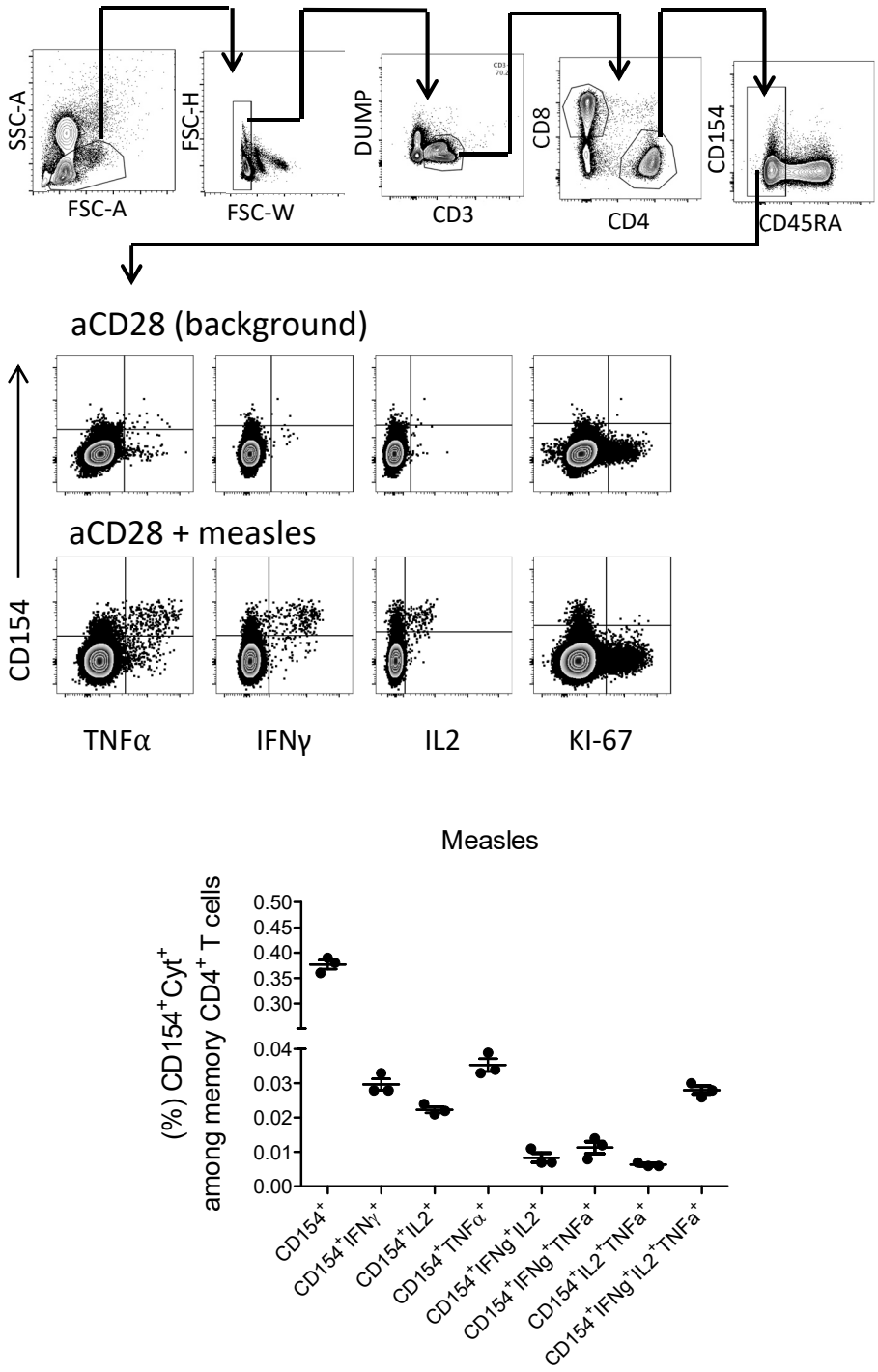

Figure 6-3: (A) Gating strategy used for analyzing antigen-reactive memory CD4⁺ lls. (B) Technical replication of antigen-reactive memory CD4⁺ T cells. Cs were separated in 3 different wells and stimulated in vitro with measles and anti- 3 for 7 h, adding Brefeldin A for the last two hours of stimulation. After in vitro antigen stimulation and ICS, we analyzed the expression of CD154, IFNγ, TNFα and IL- ll 3 replicates in order to establish limit of detection and the variance between measurements.

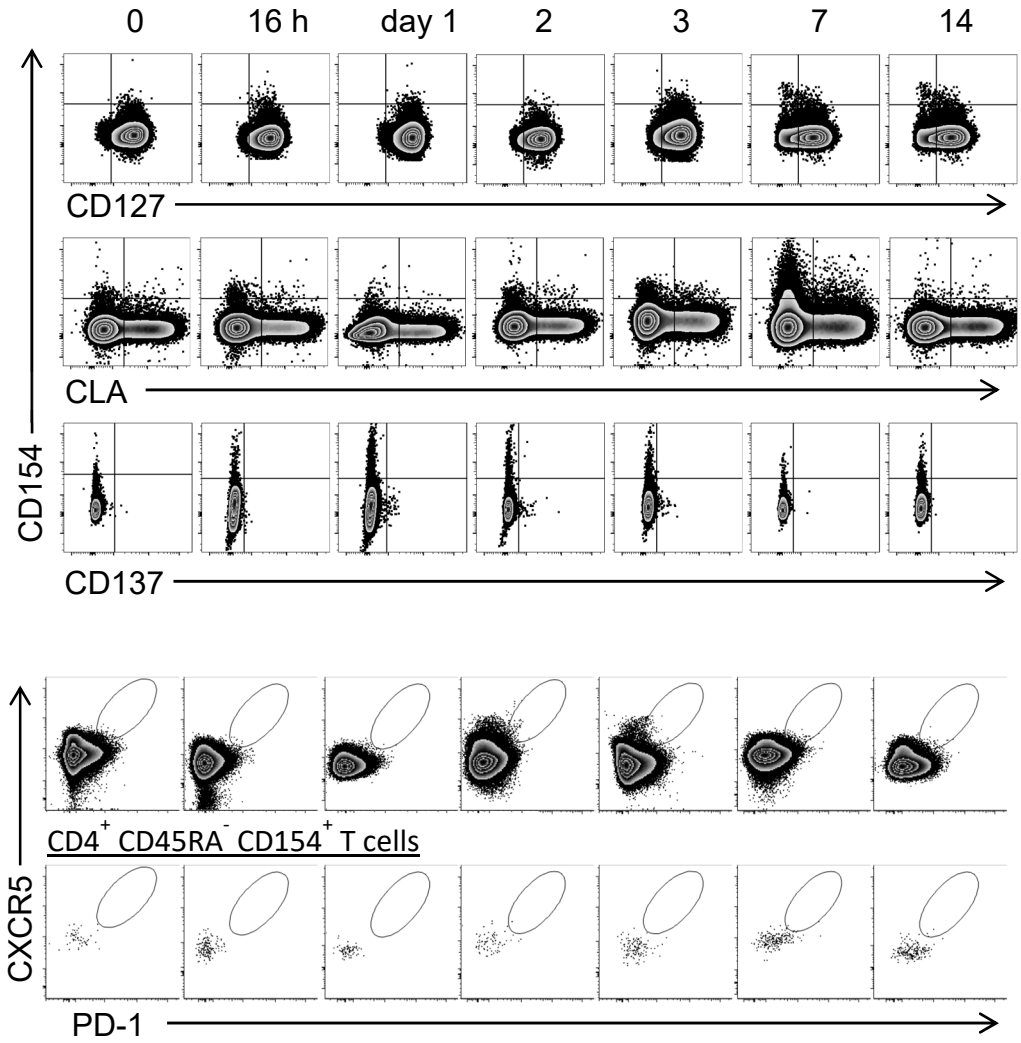

Figure 6-4. Phenotypic analysis of antigen-reactive CD4$^+$CD45RA$^-$CD154$^+$ T cells. Using ex vivo stimulation with the indicated antigens we performed a phenotypic analysis of CD154$^+$ memory CD4$^+$ T cells present in blood circulation. CD127, CLA, CD137, CXCR5 and PD-1 expression was analyzed by surface staining in antigen-memory CD4$^+$ T cells. Representative dot plots of marker expression in a representative donor at different time-points are shown.

Sample	N°cells	Replicates		UMI counts		Clonotypes		Efron Thisted	
		A	B	A	B	A	B	A	B
MMR10 d0	2500	½ RNA	½ RNA	175	269	106	150	265	393
MMR10 d1	2500	½ RNA	½ RNA	638	523	360	282	1034	815
MMR10 d14	2500	½ RNA	½ RNA	783	763	395	393	1096	1350
MMR11 d0	2500	½ RNA	½ RNA	376	399	302	314	976	967
MMR11 d1	2500	½ RNA	½ RNA	773	835	558	602	1688	1902
MMR11 d14	2500	½ RNA	½ RNA	404	463	306	343	893	958

Table 6-4: Number of cells sorted, UMI counts, clonotypes found and Efron Thisted index among MMR samples sequenced. Raw data is shown for both technical replicates of each sample analyzed.

Abbreviations and acronyms

Abbreviation / Acronym	Definition
A405... A647	Alexa Fluor™ 405... 647
Ab serum	Human male Ab serum
ANNV	Annexin V
APC	Allophycocyanin
BCL-2	B Cell Lymphoma 2
BIM	BCL-2-like protein 11
BM	Bone marrow
BMMCs	Bone marrow mononuclear cells
BSA	Bovine Serum Albumin
BV	Brilliant Violet
CCR	CC chemokine receptor
CD	Cluster of differentiation
CFSE	Caroxyfluorescein Succinimidyl Ester
CM	Central memory
CMV	Cytomegalovirus
Cy	Cyanine
DAPI	4',6-Diamidino-2-phenylindole
DC	Dendritic cell
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid

eF670	Cell Proliferation Dye eFluor 670
EL	Erytrocyte Lysis
ELISA	Enzyme-Linked ImmunoSorbent Assay
EM	Effector memory
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluoresceine isothiocyanate
HEPES	Hidroxyethylpiperazinoethanesulfonate
h	hour
ICS	Intracellular cytokine staining
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LN	Lymph node
MACS	Magnetic cell sorting
MCL-1	Myeloid cell leukemia sequence 1
MFI	mean fluorescence intensity
MMR	Measles, mumps and rubella
N	naïve
NK cells	Natural killer cells
NOXA	NADPH oxidase activator 1
PacB	Pacific Blue™
PB	Peripheral blood

PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PBE	PBS/BSA/EDTA
PCR	Polymerase Chain Reaction
PE	Phycoerythrine
Pen/Strep	100 U/ml penicillin, 100 µg/ml streptomycin
PerCP	Peridinin chlorophyll complex
PI	Propidium Iodide
PO	Pacific Orange
pp65	Human cytomegalovirus phosphoprotein 65
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SEB	Staphylococcus enterotoxin B
TCR	T cell receptor
Tfh	T folicular helper cells
TGFβ	Tumour growth factor beta
TE	Trypsin + EDTA
TNFα	Tumor necrosis factor alpha
Treg	T regulatory cells
TT	Tetanus Toxoid

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First of all, I would like to express my sincere gratitude to Prof.Dr. Andreas Radbruch for giving me the opportunity to do my PhD thesis in his group, and for allowing me to work in such interesting project. His immense knowledge and guidance helped me in all the time of research to overcome problems and progress with the study.

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Statement

Hiermit erkläre ich, die Dissertation selbständig und nur unter Verwendung der angegebenen Hilfen und Hilfsmittel angefertigt zu haben. Ich habe mich anderwärts nicht um einen Doktorgrad beworben und besitze einen entsprechenden Doktorgrad nicht. Ich erkläre die Kenntnisnahme der dem Verfahren zugrunde liegenden Promotionsordnung der Humboldt-Universität zu Berlin vom 27.Juni 2012.